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THE UNIVERSITY OF ALBERTA

A MOLECULAR GENETIC ANALYSIS OF CYTOTOXIC T CELL FUNCTION

by

CORRINNE G. LOBE

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE

OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF BIOCHEMISTRY

EDMONTON, ALBERTA

FALL, 1987

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ABSTRACT

Two cytotoxic T cell (CTL)-specific genes, referred to as B10 and C11, were isolated from a cDNA library by differential hybridization screening. In a panel of cells tested, both clones were expressed exclusively in cytotoxic T cells. Their expression was induced in activated T cells, in parallel with, but preceeding, the induction of lytic activity. DNA sequence analysis revealed that the two genes are remarkably homologous to each other and appear to encode serine proteases of novel substrate specificity. Antibodies were generated against the predicted protein product of the C11 mRNA. These were used to localize the protein to the characteristic CTL-granules, which possess the lytic activity of the cells. The correlation of expression with cytotoxic activity and localization to granules suggest that these two CTL-specific genes play an important role in target cell lysis by CTL.

The genes corresponding to the cDNA clones were isolated from genomic DNA libraries and a preliminary map of the exon/intron gene organization was deduced for C11. Regulation of B10 and C11 expression occurs at the level of transcription, therefore possible regulatory sites were identified by DNase I hypersensitive site mapping. The B10 and C11 genes were DNase I sensitive in differentiated T cells, but not in thymus or B cells. Furthermore, in CTL at least two DNase I hypersensitive sites existed in the 5' upstream region of each gene. Based on these results, a model is proposed in which the B10 and C11 genes are controlled, first by a change in chromatin conformation in differentiated T cells, then by the binding of a CTL-specific factor which leads to formation of hypersensitive sites, and finally by the binding of an inducible factor which allows transcription to proceed.

Two other related clones were identified by homology with C11. One is expressed in antigen-independent cytotoxic T cell lines and in helper T cell lines with cytotoxic activity. The other appears to be natural killer cell-specific.

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TABLE OF CONTENTS

	Page
CHAPTER I - INTRODUCTION	
A. THE IMMUNE SYSTEM	1
1. Function	1
2. Cells of the Immune System	1
3. T cell Activation	7
4. Mechanism of CTL-Induced Lysis	10
5. Immunology Needs Molecular Biology	12
6. Molecular Biology Needs the Immune System	20
B. MOLECULAR GENETICS	21
1. Control of Eukaryotic Gene Expression	21
2. Recombinant DNA Methods	34
C. FOCUS OF THIS WORK	38
BIBLIOGRAPHY	39
CHAPTER II - CLONING OF TWO GENES THAT ARE SPECIFICALLY EXPRESSED IN ACTIVATED CYTOTOXIC T LYMPHOCYTES	
A. INTRODUCTION	52
B. MATERIALS AND METHODS	54
1. Cells	54
2. cDNA Library Construction	54
3. Differential Screening	55
4. Blot Analysis	56
C. RESULTS	56
1. cDNA Library and Differential Screening	56
2. B10 and C11 are Killer Cell Specific	57

3. Blot Hybridization Analysis with B10 and C11 60

4. B10 and C11 are Induced During CTL Activation 60

D. DISCUSSION 62

BIBLIOGRAPHY 67

CHAPTER III - NOVEL SERINE PROTEASES ENCODED BY TWO CYTOTOXIC T LYMPHOCYTE-SPECIFIC GENES

A. INTRODUCTION 70

B. RESULTS AND DISCUSSION 70

C. CONCLUSIONS 76

BIBLIOGRAPHY 78

APPENDIX TO CHAPTERS II & III

A. INTRODUCTION 79

B. RESULTS AND DISCUSSION 79

1. B10 and C11 are CTL-Specific 79

2. B10 and C11 Transcripts are Sequentially Induced 84

3. B10 and C11 are Expressed in Helper T Cell Clones with Cytolytic Activity 86

4. Correlation of B10 and C11 Expression with Esterase and Cytolytic Activity 89

5. B10 and C11 are not Expressed in Peritoneal Exudate Lymphocytes 90

6. CsA and PMA do not Inhibit C11 Transcription 94

7. Localization of CCPI to Granules 98

8. Other Related Clones 99

C. CONCLUSIONS 104

BIBLIOGRAPHY 109

CHAPTER IV - ORGANIZATION AND REGULATION OF THE C11 and B10 GENES

A. INTRODUCTION 112

B. MATERIALS AND METHODS 114

1. Cell Culture 114

2. DNA Preparation 115

3. Molecular Probes and Hybridizations 115

4. Screening of Genomic Libraries 116

5. Nuclei Preparations and DNase I Treatment 116

C. RESULTS 117

1. Restriction Map of the B10 and C11 Genes 117

2. Isolation of B10 and C11 Genomic Clones 121

3. Exon/Intron Organization of the C11 Gene 124

4. B10 and C11 mRNAs are Regulated at the Level of Transcription 128

5. DNase I Sensitivity of the B10 and C11 Genes 129

D. DISCUSSION 142

BIBLIOGRAPHY 151

CHAPTER V - CONCLUSIONS 154

LIST OF TABLES

		Page
III.1	A selection of proteins that are homologous to the predicted C11 protein, CCPI	73
IIIA.1	Comparison of cytolytic activity, esterase activity and C11 and B10 expression	92

LIST OF FIGURES

		Page
I.1	Origin of cells involved in the immune response	3
I.2	Differentiation of B and T lymphocytes	5
I.3	Cellular interactions involved in the generation of a cytotoxic T cell response	9
II.1	Nucleotide sequence comparison of B10 and C11 inserts	58
II.2	Tissue specificity of expression	59
II.3	Blot-hybridization analysis of B10 and C11 expression	61
II.4	Correlation of B10 mRNA expression with cell activation in a mixed lymphocyte culture	63
III.1	Nucleotide sequence of clones B10 and C11	71
III.2	Alignment of the predicted CCPI protein sequence with other serine proteases	75
IIIA.1	Tissue specificity of B10 and C11 expression	80
IIIA.2	Northern blot analysis of B10, C11 and D12 expression	83
IIIA.3	Time-course of C11 and B10 expression	85
IIIA.4	Expression of C11 and D12 in cytotoxic T helper cells	87
IIIA.5	Northern blot of RNA from the $T_{H/K}$ cell clone, 21C11	88
IIIA.6	Expression of B10 and C11 in T cell clones with or without esterase activity	91
IIIA.7	Analysis of B10 and C11 expression in peritoneal exudate lymphocytes (PEL)	93
IIIA.8	C11 expression in CsA- and PMA-treated cytotoxic cells	95
IIIA.9	Nucleotide sequence comparison of the D12 and C11 cDNA inserts	101
IIIA.10	Tissue specificity of D12 and gN1 expression	103
IIIA.11	Model of CTL-granule involvement in killing	107
IV.1	Restriction enzyme maps of C11 and B10 cDNAs	118

IV.2	Southern blots of genomic DNA and the B10 and C11 genomic clones	119
IV.3	Restriction maps of the B10 and C11 genes	120
IV.4	Direction of transcription of gB10 and homology to C11	122
IV.5	Organization of the C11 gene	125
IV.6	Comparison of the C11 gene to other serine protease genes	127
IV.7	DNase I sensitivity of the C11 and B10 genes in MTL2.8.2 cells	131
IV.8	Appearance of C11 and B10 sub-bands due to DNase I hypersensitive sites	132
IV.9	Location of the DNase I hypersensitive sites in the B10 and C11 genes	134
IV.10	DNase I sensitivity of the B10 and C11 genes in thymus cells	136
IV.11	DNase sensitivity and hypersensitive sites in a non-cytolytic CTL line, CTL-L16	138
IV.12	DNase I sensitivity of the C11 and B10 genes in a T cell lymphoma, EL4	141
IV.13	DNase I sensitivity of the C11 and B10 genes in a B cell myeloma, S194	143

ABBREVIATIONS

ADCC:	antigen-dependent cell-mediated cytotoxicity
APC:	antigen presenting cell
BLT:	benzyoxycarbonyl lysyl thioester
bp:	base pairs
C genes:	constant genes of immunoglobulin locus
CAAT box:	eukaryotic gene promoter element with the consensus sequence 'AGCCAA(T)', located ~-70 bp upstream of the transcription start.
CAT:	chloramphenicol acetyltransferase
CCPI and II:	cytotoxic cell protease I and II, the protein products of C11 and B10, respectively.
cDNA:	complementary DNA
CGRP:	calcitonin gene related peptide
Con A:	concanavalin A
CsA:	cyclosporin A
CSF-GM:	granulocyte macrophage colony stimulating factor
CTF:	CAAT-binding transcription factor
CTL:	cytotoxic T lymphocyte
D segments:	diversity segments of immunoglobulin heavy chain locus
dscDNA:	double stranded complementary DNA
DTH:	delayed type hypersensitivity
DFP:	diisopropylflourophosphate
ER:	endoplasmic reticulum
GA:	golgi apparatus
GC box:	a 'GGGCGG' repeating element found in the promoter of many genes
GRE:	glucocorticoid response element

HAP:	heat shock activator protein
HAP chromatography:	hydroxyapatite chromatography (used to separate single from double-stranded DNA
HRE:	heat shock regulatory element
hsp:	heat shock protein
HSV-tk:	thymidine kinase of herpes simples virus
I-A:	immune antigens of the MHC complex
IFN:	interferon
Ig:	immunoglobulin; 5 classes are IgG, IgM, IgA,IgD,IgE
IL1,2,3,4,5:	interleukin 1,2,3,4,5
J segments:	joining segments of immunoglobulin gene complex
K cells:	killer cells
kb:	kilobase pairs
KLH:	keyhole limpet hemocyanin
LPS:	lipopolysaccharide
LT:	lymphotoxin
LTR:	long terminal repeat; a segment of DNA repeated at each end of the proviral form of a retrovirus
MHC:	major histocompatibility complex
MLR:	mixed lymphocyte reaction
mRNA:	messenger RNA
MT:	metallothionein
MTOC:	microtubule organizing center
MTV:	mammary tumour virus
MW:	molecular weight
NK cells	natural killer cells
ntd:	nucleotide
OVA:	chicken ovalbumin

PBS:	phosphate buffered saline pH7.2
pCTL:	precursor cytotoxic T lymphocyte
PEL:	peritoneal exudate lymphocyte
PMA:	phorbol 12-myristate 13-acetate
PMSF:	phenylmethyl sulfonyl flouride
SRP:	signal recognition particle
RHFM:	tissue culture medium containing RPMI 1640, Hepes buffer, fetal calf serum and 2-mercaptoethanol
RMCPII:	rat mast cell protease type II
T_H:	helper T cells
$T_{H/K}$:	cytotoxic helper T cells
T_K:	killer (cytotoxic) T cells
T_S:	suppressor T cells
TATA box	eukaryotic gene promoter element with the consensus sequence 'TATAAA', located 25 to 30 bp upstream of the transcription start
TCR:	T cell antigen receptor
TNF:	tumour necrosis factor
TPA:	12-O-tetradecanoyl phorbol-13-acetate; more commonly referred to as PMA
V genes;	variable genes of the immunoglobulin locus
V_H:	heavy chain variable genes

CHAPTER I
INTRODUCTION

A. THE IMMUNE SYSTEM

1. *Function*

The immune system is the body's active defense against invasion by cells or material recognized as foreign. In primitive organisms defence mechanisms exist in which self can be distinguished from non-self, but the response usually consists simply of avoidance of or, more drastically, phagocytosis of the invader. Vertebrates have developed a system much more sophisticated in its degree of specificity and manner in which the effector functions are carried out and regulated. The mode of processing stimulatory signals is more complex, and the system is able to retain a memory of antigens encountered and responds faster upon re-exposure to the antigen. This complex response requires a network of cells which interact, either directly via cell-cell contact or indirectly via secreted factors. Their collaboration serves to clear the body of environmental contaminants, viruses, parasites, bacteria or the body's own cells which have acquired foreign characteristics due to infection or malignant transformation. Thus, an understanding of how these cells function has important biological implications and clinical applications.

2. *Cells of the Immune System*

The cells which comprise the immune system are the leukocytes, or white blood cells. The leukocytes are carried by the vascular system, but are able to migrate through the walls of small venules into connective tissue spaces and lymphoid organs where they perform their functions. The lymphoid organs include the lymphatic vessels, which act as collecting ducts from the connective tissue, lymph nodes, Peyer's patches of the intestine, adenoids, appendix, tonsils and spleen.

The population of cells which make up the immune system seems to be derived from a common hemopoietic stem cell in the bone marrow (Wu *et al.*, 1968). These pluripotent stem cells give rise to progenitor cells which undergo successive commitments along distinct cell pathways, to differentiate into mature functional cells (see figure 1). A common myeloid progenitor gives rise to the granulocytes and monocytes (Abramson *et al.*, 1977), and a lymphoid progenitor develops into the lymphocytes (Jones-Villeneuve and Phillips, 1980; Schrader and Schrader, 1978).

(a) *Granulocytes*

The granulocytes, which include neutrophils, basophils and eosinophils, play a role in certain non-specific forms of defence (Spitznagel, 1977). Neutrophils are chemotactically attracted to areas of acute inflammation (a local response to cellular injury or infectious agents) and phagocytose bacteria and other particulate substances. Basophils and their connective tissue cousins, the mast cells, mediate immediate hypersensitivity, and thus intensify inflammatory reactions. When triggered by antibody bound to allergens, they release granule-associated and unstored mediators, including histamine and leukotrienes, chemotactic factors, prostaglandins and degradative enzymes. Eosinophils phagocytose antigen-antibody complexes, especially in the respiratory and digestive tracts. Major Basic Protein is released from the granules of these cells onto the surface of parasites to promote antibody-dependent killing of parasites. They also regulate allergic responses by degrading the vasoactive substances, histamine and leukotrienes, with histiminase and arylsulphatase respectively.

(b) *Macrophages*

The cells responsible for more complex immune reactions are the macrophages and the lymphocytes. Macrophages are derived from monocytes, which in turn are thought to arise from the same myeloid progenitor as the granulocytes (Abramson *et al.*, 1977). They

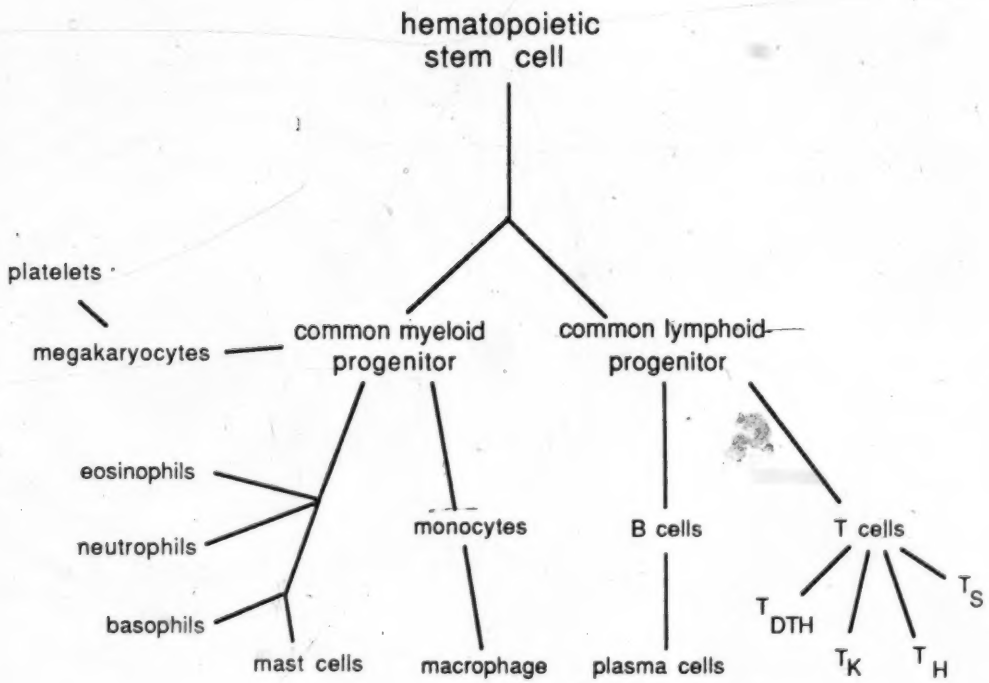

FIGURE I.1 Origin of cells involved in the immune response. A common pluripotent stem cell gives rise to distinct progenitor cells, one for myeloid and the other for lymphoid cells. The myeloid progenitor gives rise to the "committed" cells shown. The lymphoid progenitor has the potential to differentiate into either B or T cells.

are highly motile phagocytes, able to swallow entire cells (often several at a time), bacteria, protozoa and inorganic substances. The cells can carry out either non-specific phagocytosis or more specific reactions via serum proteins (opsonins) which coat the target surface, such as IgM or IgE immunoglobulins or the C3 component of complement. In the latter case, specific Fc or C3 receptors are utilized, which promote attachment and ingestion (Unkeless *et al.*, 1981; Rabellino *et al.*, 1978). Macrophages play an important role in humoral and cell-mediated immune responses (Adams and Hamilton, 1984). Generally, this is accomplished by trapping and ingesting the antigen, processing it in some way (probably partial digestion), and presenting the processed antigen to lymphocytes (in association with class II MHC molecules, as discussed later). Simultaneously they produce soluble factors which drive lymphocyte proliferation. They also secrete a variety of other immunoregulatory proteins, complement components, and other factors involved in cellular regulation or microbe destruction.

(c) *Lymphocytes*

There are two major classes of lymphocytes, B and T, named according to their site of maturation (see figure 2). Pre-B lymphocytes mature in the **B**ursa of Fabricius in birds and the "**B**ursa-equivalent" (bone marrow, spleen and liver) in other animals, into mature B cells which express antigen-specific immunoglobulin receptors on the cell surface. When antigen is recognized by a B cell via its receptor, in the presence of factors produced by macrophages or T lymphocytes, the cell becomes activated into a plasma cell. It subsequently secretes antibodies which have the same specificity as its surface receptors (Warner, 1974). The secreted antibodies enter the blood and other bodily fluids to defend against the extracellular phases of bacterial and viral infections -- therefore this is referred to as the humoral branch of the immune response. If they then encounter antigen, the humoral antibodies bind to the antigen or antigen-bearing foreign material. This interaction

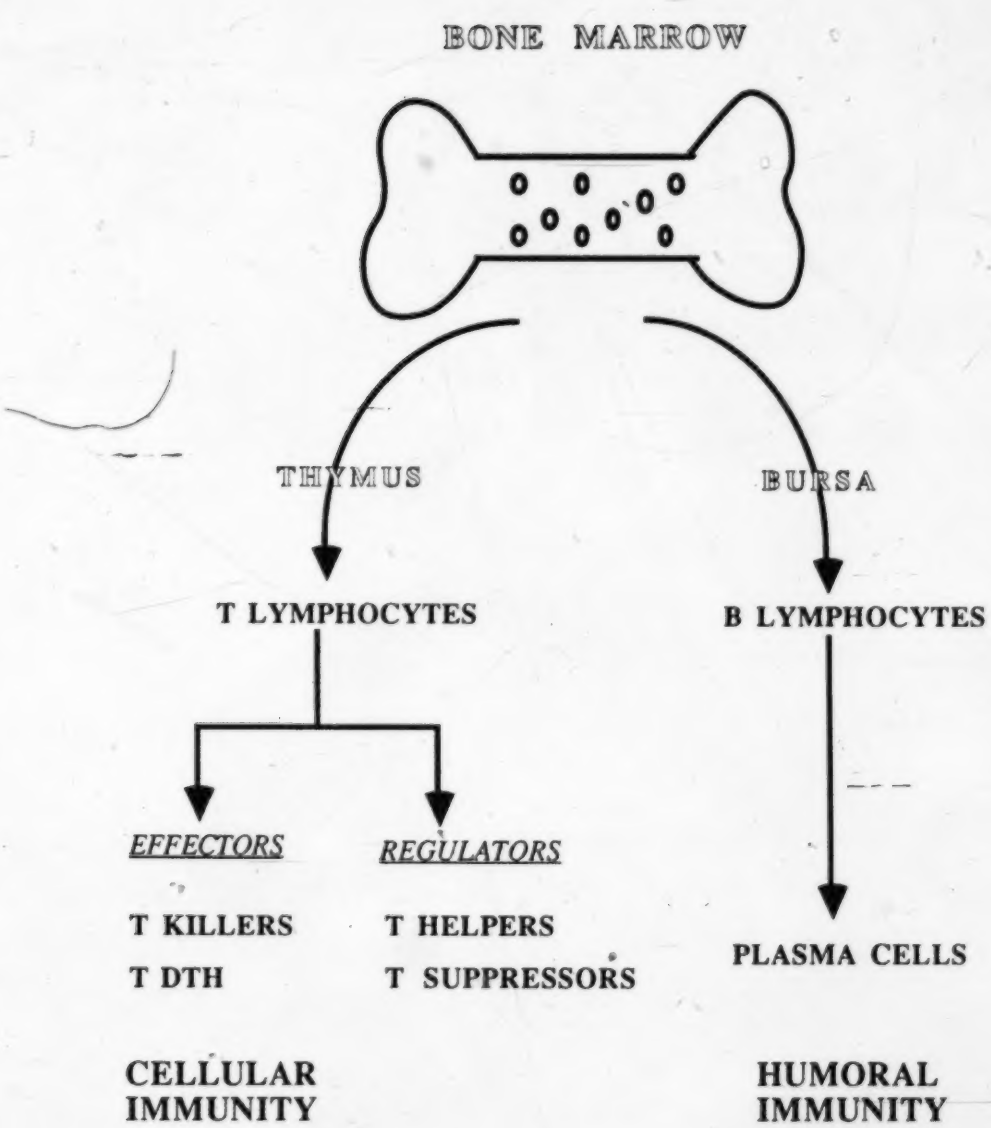

FIGURE I.2 Differentiation of B and T lymphocytes. Lymphoid progenitors from the bone marrow differentiate into T cells in the thymus, or B cells in the bursa-equivalent (foetal liver, bone marrow). The B lymphocytes further differentiate into plasma cells upon antigen stimulation. The effector T cells carry out the destruction of target cells. The regulatory T cells produce lymphokines which modulate the action of effector T and plasma cells. Recognition and destruction of foreign cells (cellular immunity) is carried out by T cells, whereas B cells are responsible for clearing soluble antigens (humoral immunity).

activates the pathways for elimination of the antigen, for example phagocytosis by macrophages via their Fc receptors, or lysis by complement (Reid and Porter, 1981).

Cells destined to become T lymphocytes migrate from the bone marrow to the thymus, the major site of T cell maturation (Scollay *et al.*, 1984). Here, under the influence of thymic hormones and epithelial cells, the pre-T cells express the T cell antigen receptor (TCR) on their surface. T cells which leave the thymus migrate to the lymphoid organs and enter the pool of circulating lymphocytes. Upon interaction with antigen, they are driven through the final steps of maturation to become mature T cells. Their defence is primarily against fungi, parasites, intracellular viral infections, cancer cells and foreign tissue. Consequently, this is known as the "cellular immune response".

Two main classes of T cells have been defined (Cantor and Boyse, 1975) according to their function upon antigen interaction: regulatory T cells [helper (T_H) and suppressor (T_S) T cells] and effector cells [cytotoxic T cells (T_K or CTL) and delayed-type hypersensitivity cells (DTH)]. When helper T cells recognize antigen, via their T cell receptor, they produce soluble factors (lymphokines) which act on other cells in the immune system, and thereby potentiate both humoral and cell-mediated immune responses. Suppressor T cells are not well defined, but seem to inhibit the functions of other lymphocytes, either directly or via soluble factors. When cytotoxic T cells recognize cell surface antigens, such as viral or tumour antigens, they destroy the target cell by causing it to lyse. DTH cells secrete factors which attract a variety of inflammatory cells (eg. macrophages).

It is the T lymphocytes which are of primary interest to this study. The activation and function of these cells will be discussed in greater detail in section 3.

(d) *NK and K cells*

Natural Killer (NK) cells are bone marrow derived cells which exhibit cytotoxic activities similar to CTL and can be stimulated by IL2 and gamma-interferon (Ortaldo and

Herberman, 1984). They display low levels of the T cell marker, Thy1, as well as some markers thought to be NK-specific (NK1, Qa-4 and Qa-5; Tai and Warner, 1980; Koo and Hatzfield, 1980) and receptors for the Fc portion of IgG molecules (Perussia *et al.*, 1983). The efficient anti-tumour activity of these cells was the first evidence for their existence, but they can also kill bone marrow and a few other normal cells (Ortaldo and Herberman, 1984; Nunn *et al.*, 1977)). What target cell molecules they recognize and the method of recognition are not known, but unlike CTL, their cytotoxic activity is not MHC-restricted (Herberman *et al.*, 1975). A clonal population can kill a variety of tumour targets, but since the antigen they recognize is not known, it isn't obvious if their action is specific or not. As will be discussed for CTL, the probable effector molecules of lysis are contained in the cytoplasmic granules (Henkart *et al.*, 1984). The cells occur in spleen, lymph node, peripheral blood, and peritoneum. Their pre-dilection to destroy tumour cells and activation by gamma-interferon suggests these cells play a role in the body's natural resistance to tumours, although this is not certain. Whether NK cells are simply related to T cells, or represent a pre- or post-T cell population which do not use the antigen-specific TCR is still a contentious issue.

Killer (K) cells are characterized by their ability to kill antibody-coated target cells without previous exposure to the antigen and without the participation of complement. This phenomenon is known as antigen-dependent cell-mediated cytotoxicity, or ADCC. K cells possess receptors for the Fc portion of IgG, as do NK cells. It is likely that K and NK cells are a single population of cells, with varying efficiences for ADCC or NK activity.

3. *T cell activation*

The focus of the work that will be described is on the T cells, and in particular, regards the activation and function of the cytotoxic T cell subset. Most T cells are in an inactive or precursor state. The dual signal of antigen and a lymphokine triggers them to

synthesize new RNAs and proteins, increase in size (blast transformation), and eventually proliferate. As mentioned, foreign antigens from the surface of a stimulator cell are recognized via the antigen-receptor of the T_H cell (figure 3; Paetkau *et al.*, 1980). This recognition is in association with syngeneic class II MHC antigens expressed by the antigen presenting cells, such as macrophages or B cells. Macrophages produce interleukin 1 when they encounter antigen. The dual signal of antigen and interleukin 1 induces the T_H cell to synthesize and secrete a variety of lymphokines (Moller, 1985). These factors are generally glycoproteins which stimulate growth and differentiation of many types of hematopoietic cells and include colony stimulating factors for granulocytes and macrophages (CSF-GM), interleukin 3 (IL3), interferon-gamma, interleukin 2 (IL2), and a variety of B cell growth and differentiation factors.

Concurrently, a precursor cytotoxic T cell (pCTL) recognizes antigen on the surface of "self" cells, which are distinguished via syngeneic class I MHC molecules expressed on almost all somatic cells. This recognition is again via the T cell antigen receptor. When the pCTL is initially stimulated with antigen, there is a transient burst of transcription and a subsequent synthesis of new proteins, including one subunit of the IL2 receptor, the transferrin receptor, c-*myc* and MHC molecules (Teshigawara *et al.*, 1987; Kronke *et al.*, 1985; Neckers and Cossman, 1983; Kelly *et al.*, 1983; Cotner *et al.*, 1983). Up-regulation of the transferrin receptor is necessary since transferrin is a universal requirement for long-term growth of all mammalian cell types (Cotner *et al.*, 1983). The protooncogene c-*myc* is a nuclear DNA binding protein which seems to be involved in the transit of cells from the G_0 to the G_1 phase of the cell cycle (Persson and Leder, 1984). The newly synthesized subunit of the IL2 receptor associates with the subunit already on the cell surface. Together, the subunits have a high affinity for IL2, while the constitutive chain alone comprises the low affinity receptor (Wang and Smith, 1987; Rusk and Robb, 1987). The high affinity receptor on the pCTL allows binding of the lymphokine, produced by the neighboring T_H. This induces progression to the second stage of cell

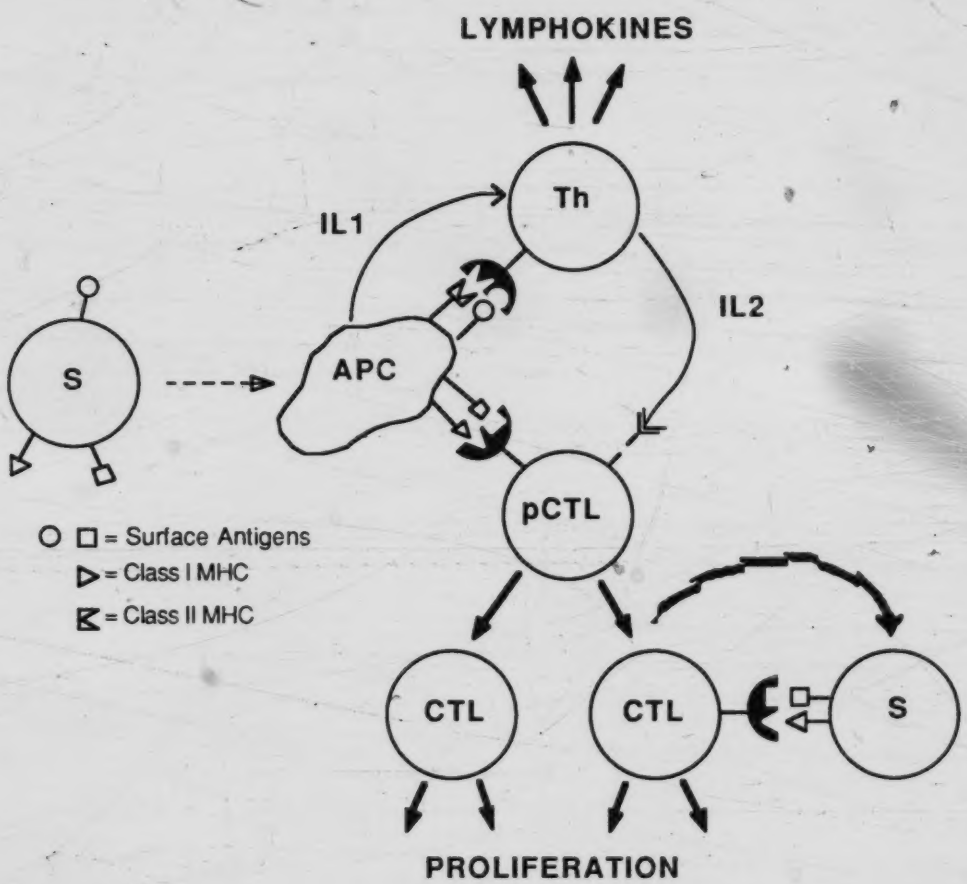

FIGURE I.3 Cellular interactions involved in the generation of a cytotoxic T cell response. Surface antigens from a stimulator cell (S) are processed by an antigen presenting cell (APC) and recognized by the antigen receptors on helper T lymphocytes (Th) and precursor cytotoxic T lymphocytes (pCTL). The resultant CTL is capable of lysing any antigen-bearing target cell which it then encounters. See text for explanation of signalling events.

activation in which DNA synthesis takes place and the cells proliferate into a clonal population of cytotoxic cells expressing the same specific T cell antigen receptor (Lowenthal *et al.*, 1985). These cells are now competent to bind and lyse target cells.

4. *Mechanism of CTL-Induced Lysis*

The essential changes a pCTL undergoes to become a killer cell and the mechanism by which it then lyses its target are not known in any molecular detail. Studies using metabolic inhibitors, antibodies against cell surface proteins, and observations of morphological changes as the CTL carries out its function enable some general deductions to be made about the mechanism of lysis (Berke, 1983; Nabholz and MacDonald, 1983). The lytic reaction requires direct target cell contact and is unidirectional -- the effector cell comes away uninjured, and in fact can kill more than one target cell (Zagury *et al.*, 1975). Binding of target cells is Mg^{2+}-dependent and requires energy. The cells come into close proximity and the plasma membranes interdigitate at the contact region. Initial Ca^{2+}-independent steps occur upon target conjugation, which can be blocked by inhibitors of methylation or compounds which alkylate cell-surface thiol groups. This is followed by Ca^{2+}-dependent steps, in which serine proteases may play a role (Redelman and Hudig, 1983).

Within 30 minutes after binding, the microtubule-organizing center (MTOC) and Golgi apparatus (GA) of the effector cell are reoriented proximal to the contact area (Kupfer and Dennert, 1984). This observation suggests a delivery of cytotoxic substances from secretory vesicles of the GA, since membrane-inserted and secreted cell products are processed and packaged in that organelle. One possible molecule involved in target lysis was originally discovered by electron microscopy of CTL and lysed target membranes (Dennert and Podack, 1983). Lytically active CTL contain characteristic dense granules. Upon stimulation with antigen or Concanavalin A (ConA), proteins which appear to originate in these granules polymerize to form two types of tubular structures distinguished

by their diameters (160 and 50 A), designated poly P1 and poly P2. These "polyperforins" are transferred to the membrane of the target cell, resulting in trans-membrane channel formation similar, but different in size, to complement lesions. In fact, Podack and Konigsberg (1984) demonstrated that isolated CTL granules are capable of Ca^{2+}-dependent cytolysis with comparable or higher activity than, but without the target specificity of, the cells they were isolated from. Similar observations have been made using NK cells, in which the pore-forming proteins were referred to as cytolysins (Henkart *et al.*, 1984). Membranes of target cells lysed by granules show the characteristic donut-shaped lesions of the same dimensions as poly P1 and poly P2. This is analogous to the final event in complement-mediated cytolysis (Reid and Porter, 1981), in which C9 polymerizes to form disulfide-linked tubular structures which lyse targets by membrane insertion and channel formation. However, one event known to accompany cell-mediated but not complement- or perforin-induced lysis is the fragmentation of target cell DNA (Duke, 1983). This suggests that the polyperforins act as transmembrane channels for toxic molecules to be passed from the granules of the effector to the target, where they can inflict their damage.

A potential toxic molecule which is in CTL-granules is lymphotoxin (LT; Eardley *et al.*, 1980). Lymphotoxin has slow cytotoxic effects when incubated with cells, however, under conditions in which target cells are induced to take up this substance, lysis occurs much faster. In addition, cytolysis by LT is accompanied by fragmentation of target cell DNA (Schmid *et al.*, 1986), making it a prime candidate for a toxic molecule which may pass into the target cell via polyperforin channels. The other granular molecules which have been characterized are proteoglycan and serine proteases. It is likely that the former of these acts as a carrier molecule for the latter or may play a role in protecting the killer cell from its own cytolytic machinery (MacDermott *et al.*, 1985). The role for the granular serine proteases is unclear.

5. *Immunology needs Molecular Biology*

Although the studies to date have defined the stages involved in T cell differentiation and function, many questions remain regarding the precise molecular identity of the individual components involved. B and T cells are derived from a common precursor, but the stimuli which are responsible for directing the cell along a particular differentiative pathway, the molecules which are required to respond to that stimuli, and the products which are expressed as a result are unclear. T cells have been divided into subclasses according to function, and each of these subclasses usually displays a characteristic set of cell surface antigens (Cantor and Boyse, 1977). In mouse, for example, helper T cells express the surface marker, Lyt1, while cytotoxic and suppressor T cells instead display the Lyt 2 and 3 surface proteins. However, these markers are usually of unknown relevance to the functions of the cells on which they are expressed. Indeed, this categorization of T cells is probably an oversimplification, since T cell clones have been identified which display functions of more than one subclass (Tite and Janeway, 1984). More useful cell markers would be molecules which are known to be required for helper, cytotoxic or suppressor function.

Such elements can be readily identified and characterized using recombinant DNA techniques to isolate new, functionally relevant molecules. In addition it may be possible to elucidate the functions of known markers through the application of molecular genetic techniques. The sequence of the cDNA clone can be determined, providing information on the primary structure of the molecule. This can be an important clue as to its function if the protein sequence is homologous to other characterized molecules. Expression of the clone allows synthesis of relatively pure material for biological assays. Site specific mutagenesis coupled with transfection experiments permit manipulation of the molecule to study its function. Many examples already exist in which a molecular genetic approach has increased our knowledge of how the immune system functions. Some of these examples are described below.

(a) *Immunoglobulins*

The best characterized molecules of the immune system are the B cell antigen receptor and its secreted form, the immunoglobulins. These molecules are made up of two identical light chains and two identical heavy chains joined by disulphide bonds (Gally, 1973). Each chain has an N-terminal variable region which is involved in antigen binding, and a constant region. The constant region of the heavy chains, from which the Fc fragment is derived, triggers effector functions such as complement fixation.

B cells exhibit a seemingly endless repertoire of antigen specificity. Characterization of the immunoglobulin genes provided the explanation for how diversity is generated (Adams, 1980; Gough, 1981; Tonegawa, 1983): Each chain is encoded by a relatively large genetic locus which is made up of several V genes which encode the variable region of the chain, and C genes which encode the constant domain. In addition, a set of J (joining) segments in the case of the light chain genes, and D (diversity) and J segments in heavy chain genes, lie between the set of V genes and set of C genes. During the process of differentiation of B lymphocytes, these genes undergo rearrangements, which are independent of antigenic stimulation (Gough, 1981). For the light chain genes this involves joining any one of the V genes to a J segment, whereas with heavy chain genes there is first a V to D joining and then a D to J joining. In each case, the intervening DNA is deleted out (Hozumi and Tonegawa, 1976). There is some sloppiness to this joining, that is, when a particular V gene joins a given J segment, the joining will not always occur at the same nucleotides (Tonegawa, 1983). Therefore the incredible diversity of immunoglobulin specificity arises because there are many possible combinations of V genes with D and J segments, because of the sloppiness of these junctions and because two of these recombined gene products give rise to the final molecule (heavy and light chains).

Analysis of the immunoglobulin gene complex also explained two other observations, namely heavy chain class switching and the existence of membrane and secreted forms of antibody with the same specificity. There are five major classes of

immunoglobulin molecules (IgG, IgM, IgA, IgD, and IgE) which mediate different biological activities, and each of these is represented by a separate cluster of C genes. The heavy chain class switching is due to a second type of rearrangement which occurs after antigenic stimulation in which the expressed V_H gene is reassociated with a different C_H gene (Davis *et al.*, 1980). Thus, the class (and hence biological activity) of the antibody produced is changed, but the antigen specificity determined by the first rearrangement is retained.

The cell surface receptor, which has the same antigen specificity as the secreted antibody, is produced from the same gene (Early *et al.* , 1980). At the 3' end of the C mu heavy chain gene is a coding segment that specifies a hydrophilic carboxy terminus used by the secreted form. Further downstream are another two exons which encode a carboxy terminus with the hydrophobic characteristics of a transmembrane polypeptide, used by the membrane form. To produce the cell surface receptor, all of the exons are transcribed, but the exon encoding the hydrophilic carboxy terminus is spliced out during post-transcriptional processing. This leaves an mRNA which encodes protein with a hydrophobic trans-membrane carboxy terminus. To produce the secreted form the last two exons are not transcribed, so the exon encoding the hydrophilic carboxy terminus is used. Thus, differences in the termination sites used and in post-transcriptional processing give rise to the two forms of immunoglobulin.

(b) *T Cell Antigen Receptor*

The cloning of the T cell antigen receptor genes (Hedrick *et al.*, 1984; Yanagi *et al.*, 1984; Saito *et al.*, 1984) has confirmed that T cells use a method of rearrangement of gene components analogous to B cells. The T cell receptor is composed of a disulfide-linked alpha and beta chain, which are encoded by genes with V,D,J and C elements (Saito*et al.*, 1984; Chien *et al.*, 1984). These genes are very like the immunoglobulin genes in sequence, patterns of rearrangement and the sizes and position of the different gene

segments (Chien *et al.*, 1984). Although the answer is not yet clear, the availability and expression of cDNA clones of the TCR should finally settle the question of "One receptor or two?" - how T cells recognize antigen in the context of MHC molecules.

(c) *Major Histocompability Complex (MHC)*

The murine MHC gene complex has been well characterized. This has provided a clear picture of the structure of type I (expressed by somatic cells) and type II (expressed by cells of the immune system) molecules (Hood *et al.*, 1983). The class II molecules are encoded by the I region in the MHC, which encodes two cell-surface glycoproteins, I-A and I-E. Each is made up of two polypeptide chains designated alpha and beta. They are highly polymorphic between individuals, thereby allowing a distinction between cells which are self and non-self. The antigen-specific receptors of regulatory T lymphocytes recognize foreign antigen only in association with self I-A molecules on the surface of antigen presenting cells (APC).

Transfection and expression of the cDNA clones has been useful in the analysis of the features of an antigen presenting cell which are necessary to trigger a T cell response (Germain and Malissen, 1986). Malissen *et al.* (1984) transfected genes encoding I-A molecules of the k haplotype (A^k-alpha and A^k-beta) into mouse L cell fibroblasts and hamster B cell lines. Both the non-lymphoid L cells and lymphoid B cells expressed the class II molecules correctly, as judged by serological analyses and two dimensional gel electrophesis. The transfected cells were then tested for their ability to present the antigens keyhole limpet hemocyanin (KLH) and chicken ovalbumin (OVA) to I-A^k restricted T cell helper hybridomas specific for these antigens, as measured by IL2 production by the T_H. All of the L cell and B cell transformants were capable of efficiently presenting the KLH antigen. In fact, the level of IL2 produced by the T_H in response was proportional to the level of I-A^k molecules expressed on the surface of the transfectants. This indicates that surface-expressed I-A^k molecules are sufficient to confer antigen-presenting capabilities.

However, in the case of the OVA antigen, the transfectants failed to act as APC, whereas a murine I-A^k positive B cell is able to present this antigen to the I-A^k helper hybridomas. It may be that this antigen needs to be enzymatically or chemically modified by the APC, a process not carried out by the transfectants. Another possibility is that other accessory surface molecules are required, in addition to class II molecules, for certain antigens. This can be further tested by screening other antigens or derivatives of them, and co-transfecting I-A genes with other molecules found on the surface of APC, such as LFA-1 or L3T4 (Wilde *et al.*, 1983).

(d) *Lymphokines*

Many lymphokine genes have now been cloned: Interleukin 1 (IL1), a lymphocyte-activating factor which drives proliferation and is produced by macrophages (Lomedico et al., 1984; March *et al.*, 1985; Auron *et al.*, 1984); Interleukin 2 (IL2), an important T cell growth factor which is one of the requirements for the proliferation of activated CTL (Taniguchi *et al.*, 1983; Kashima *et al.*, 1985); GM-CSF, a factor which stimulates the growth of macrophages and granulocyte colonies (Gough *et al.*, 1984; Lee *et al.*, 1985; Wong *et al.*, 1985); Interleukin 3 (IL3), which also induces hematopoietic precursor cells to produce colonies of macrophages and granulocytes, and stimulates B and T cells (Metcalf, 1986; Fung *et al.*, 1984; Yokota *et al.*, 1984); the anti-viral agent, gamma-interferon, which stimulates the activity of macrophages (Gray and Goeddel, 1983); interleukin 4 (IL4), also known as B cell stimulatory factor, which promotes the differentiation and proliferation of B cells (Lee *et al.*, 1986; Noma *et al.*, 1986) and interleukin 5 (IL5), also known as T cell replacing factor, which is important in maturation of activated B cells into antibody-secreting cells (Kinashi *et al.*, 1986).

These clones have been used as probes to monitor expression of the corresponding lymphokine genes, providing a functional assay for cells capable of potentiating an immune response, i.e. helper T cells (Mosmann *et al.*, 1986). Expression of recombinant

lymphokines has allowed a more definitive characterization of their activities, as opposed to analyzing the activity of "partially purified" supernatants of helper cell lines (Lomedico *et al.*, 1984; March *et al.*, 1985; Taniguchi *et al.*, 1983; Metcalf, 1985). Recombinant material has potential clinical use in boosting immune responses against disease or tumour cells (Rosenberg *et al.*, 1986). The cDNA clones also permitted analysis of how cyclosporin A (CsA), an immunosuppressive drug used therapeutically following tissue transplantation, exerts its effect. CsA blocks the production of lymphokines in helper T cells. Using lymphokine cDNA clones as probes, this blockage was found to correlate with the absence of the mRNAs encoding the lymphokines (Elliot *et al.*, 1984). Since lymphokines play a central role in immune responses, it appears that at least part of the immunosuppressive effect of CsA is due to its selective inhibition of lymphokine mRNA accumulation, either directly or by inhibiting molecules which would activate transcription.

(e) *IL2 Receptor*

The IL2-receptor is up-regulated in CTL when antigen is recognized, for utilization of the second activation signal of IL2. Two classes of receptor for IL2 have been demonstrated which possess either high or low affinity for the ligand (Robb *et al.*, 1984). Antigenic stimulation increases the number of cell-surface high affinity receptors, and it is this class which is necessary for IL2 to carry out its biological effects. A cDNA clone for the IL2 receptor gene was isolated, but when it was transfected into cells, it produced only the low affinity activity. This implied that the receptor must consist of another element. By cross-linking studies, another chain of the IL2 receptor has now been detected. Each chain is able to bind IL2 with low affinity, but together they form the high affinity receptor. This explained why Tac-negative resting T cells can bind IL2, since one of the chains (the second protein identified) is constitutively produced, whereas expression of the other chain (Tac) requires antigen and low levels of IL2 to induce its expression, and thus produce a high affinity receptor.

(f) *T Cell Surface Markers*

Many T cell surface marker genes have recently been cloned. One of these, the CD3 complex (T3) appears during intrathymic ontogeny in correlation with the acquisition of immunological competence by developing thymocytes (Reinherz *et al.*, 1983). Characterization of the CD3 complex using monoclonal antibodies showed that it is associated with, and may be required for, surface expression of the TCR. This has led to the postulation that the proteins of the CD3 complex are involved in the signal transduction that follows antigen recognition by the TCR. The genes encoding two of the three chains of human CD3, epsilon and delta, have now been cloned (van den Elsen *et al.*, 1985a; Gold *et al.*, 1986). The sequence of these cDNAs and homologies between them suggest a possible mechanism for interaction with the T cell receptor. The putative transmembrane domain contains an Asp residue, and of the neighbouring 23 residues toward the N-terminus, 6 are charged and 9 are shared between the epsilon and delta chains. These characteristics are also conserved between the human and mouse delta chains, as revealed by cDNA sequence (van den Elsen *et al.*, 1985a; van den Elsen *et al.*, 1985b; Gold *et al.*, 1986). The alpha and beta chains of the TCR both contain a Lys in their transmembrane domains, which could form salt bridges with the charged residues of the CD3 chains, in particular with the conserved Asp residue. These interactions would be particularly significant in the hydrophobic membrane environment. Site-specific mutagenesis of the regions encoding the transmembrane domain and transfection of the CD3 and TCR genes can be used to test this model.

Other recently cloned T cell markers include the human CD2 and CD4/CD8 molecules (Parnes, 1986), murine Lyt 2 (Zamoyska *et al.*, 1985) and Thy1 (Moriuchi *et al.*, 1983; Giguere *et al.*, 1985; Van Rijs *et al.*, 1985). As in the case of CD3, availability of the DNA clones will allow sequence and expressional analysis to determine their relevance, if any, to T cell function.

(g) *Lymphotoxin and Tumour Necrosis Factor*

Two genes encoding factors which are associated with tumour cell killing have been cloned and expressed in E.coli (Gray *et al.*, 1984; Pennica *et al.*, 1984). Lymphotoxin (LT) is a cytotoxic factor produced by lymphocytes (Ruddle, 1985). A synthetic gene encoding lymphotoxin (LT) was made, based on the protein sequence, in order to produce recombinant LT, and using this gene a cDNA clone was isolated (Gray *et al.*, 1984). Expression of two versions of the synthetic gene in E. coli and analysis of the products' cytotoxic activity showed that 16 residues at the carboxy terminus are necessary for its function. Experiments in which mice were injected with the recombinant material showed that LT causes significant necrosis of methyl cholanthrene-induced sarcomas *in vivo*. Tumour necrosis factor (TNF) is another cytotoxic factor, with properties similar to LT, which is produced by activated macrophages. Sequence of the cDNA clone revealed that TNF is translated with a large 76-residue leader sequence, probably required for secretion. This factor was also expressed in E. coli and the recombinant material exhibited necrotic activity *in vivo* on methyl cholanthrene-induced sarcomas (Pennica *et al.*, 1984). cDNA cloning of these factors demonstrated that they are, in fact, two distinct molecules. Their similar activies can be explained by comparison of the sequences, which revealed that they are 30% homologous at the amino acid level. There are three regions which are highly conserved, one of which is the carboxy terminus shown to be requred for functionally active lymphotoxin. Using the cDNA clones as probes, the cells which produce each of these factors can now be identified. Production of recombinant material in E. coli of both these factors permits the generation of otherwise unavailable material to use in assessment of the biological activities of both LT and TNF, differences between them and their clinical potential (Gray *et al.*, 1984).

6. *Molecular Biology needs the Immune System*

From the examples above, it is apparent that many questions regarding how the immune system functions can be answered using a molecular biological approach. On the other hand, the cells of the immune system, and in particular the lymphocytes, offer an ideal model in which to study gene regulation. To reach a functional state, these cells must differentiate from common progenitor cells to cells whose fate is determined (eg. pre-B or pre-T cells) and then to mature cells. Finally, they must be activated by external signals (antigen and lymphokines) to carry out their function. Each of these stages is a reflection of changes in chromatin structure and gene expression.

Cell lines at various stages of differentiation are available. Bone marrow cells grown in Dexter cultures or immortalized by viral transfection have been used to study cells at the early stages of differentiation (Dexter and Lajtha, 1974; Whitlock and Witte, 1981). The adaptation of culture conditions for the growth of T-lymphocytes in long-term culture (Fathman and Fitch, 1982) offers a clonal source of T cells which can be classified as helper, cytotoxic or suppressor. Myeloma and hybridoma cell lines provide clonal populations of B cells, all expressing the same antigen receptor (Kohler and Milstein, 1975; Bankert *et al.*, 1978). Both T and B cells grown in culture can be stimulated, either by lymphokines and the correct antigen (if the specificity of the T or B cell receptor is known), or non-specifically with a mitogen or lectin (eg. ConA for T cells, LPS for B cells). Therefore, one can mimic an immune response using a clonal population of cells *in vitro* . The network of immune cells thus represents a microcosm of cell differentiation and activation, in which many of the individual elements are available for study and can be manipulated to look for consequent changes in gene structure and expression.

B. MOLECULAR GENETICS

1. *Control of Eukaryotic Gene Expression*

The phenotype of a cell is determined by the genes which it expresses. Normally every cell contains the full repertoire of genes, but differences in the level of expression of the genes establish the wide variety of cell types.

Chromosomes exist in the nucleus of a eukarytic cell in a highly condensed form, complexed with proteins. The chromatin around genes which are actively transcribed is decondensed, (Weisbrod, 1982) thereby allowing RNA polymerase, together with other transcription factors, to bind to the DNA (Elgin, 1982). Hypomethylation of important nucleotides in the gene may also occur, although this issue is still controversial (Bird, 1984; Meijlink *et al.*, 1983). Once the higher order structure of a gene has changed to make it available for transcription, the promoter region and regulatory proteins which bind to this region become important in determining the efficiency and specificity of transcription. The primary transcript produced, which includes exons and introns (*ex* pressed and non-coding *in* tervening sequences) is polyadenylated and capped at the 3' and 5' termini, respectively (Perry, 1981). This transcript, as a complex with proteins and short RNAs, is then processed by the removal of introns and the mature RNA is transported to the cytoplasm where it is translated into the corresponding protein. In the cytoplasm, the half-life varies between different RNAs, due at least in part to RNases which recognize specific sequences in the mRNA (Brawerman, 1987). Thus, gene expression can be controlled at many levels: chromatin structure, modified nucleotides, initiation of transcription, post-transcriptional processing, translation and post-translational processing. Each of these is described in detail below.

(a) *Cell Differentiation and Chromosome Structure*

As pluripotent stem cells differentiate into a specific cell type, certain genes become available for transcription, due primarily to changes in chromatin structure. This change in chromatin structure can be demonstrated with pancreatic DNase I, which at high enough concentrations will digest all of the chromosomal DNA, but at low concentrations only degrades the sequences which are transcribed. For example, globin genes are preferentially digested by DNase I in erythrocyte nuclei, but not in fibroblast nuclei (Weintraub and Groudine,1976) and ovalbumin genes are sensitive in oviduct nuclei but not in liver cell nuclei (Garel and Axel, 1976). Similarly, in pre-B cells which have rearranged and express their heavy chain loci but not their light chain loci (heavy chain rearrangement and expression precedes light chain), the heavy chain gene region is much more sensitive to DNase I than the light chain gene region (Yancopoulos *et al.*, 1986). In the same experiments, the T cell antigen receptor gene region was found to be relatively resistant to DNase I in B cells . Interestingly, even genes which are transcribed only a few times per cell generation are nuclease sensitive, indicating that the decondensation is a prerequisite for transcription, but not all decondensed DNA is being transcribed (Garel *et al.*, 1977; Wu, 1980).

A more specialized example of the use of structural alterations to control gene expression is seen in differentiating B cells. As already described, the immunoglobulin genes become transcriptionally active when they rearrange to juxtapose V and C genes. The heavy chain genes rearrange first, and their expression signals the light chain genes to rearrange. The pre-B cell becomes a mature, immunocompetent B cell when the genes for both chains are rearranged and expressed, producing a functional antigen receptor.

(b) *Transcriptional Control*

Many differentiated cells can be transiently activated by external stimuli. This activation corresponds to the induction of transcription of a specific set of genes to carry

out the cell's response to the stimuli. Probably the simplest mechanism by which an external signal can induce gene activation is used by the steroid hormones. Steroid hormones bind to high affinity receptor proteins in the cytoplasm of responsive cells, thereby causing a change in the conformation of the receptor, after which the hormone-receptor complex passes into the nucleus and binds to specific sites on the DNA to activate gene transcription (Grody *et al.*, 1982). In the majority of cases not involving steroid hormones, however, the stimulatory signal or signal molecule does not act directly on the gene; instead an intermediate messenger(s) must be postulated. Whether by a primary signal or a secondary messenger, gene activation is effected by specific DNA-protein interactions which occur at the promoter region or enhancer elements, as discussed below.

Whereas the entire coding region of a transcriptionally-active gene is DNase I sensitive, the promoter and enhancer regions display DNase "hypersensitivity". Finer resolution of hypersensitive sites is afforded by "footprinting" techniques, in which a sequence of DNA containing the regulatory elements is incubated with a nuclear protein extract and then treated with low levels of DNase I (Emerson *et al.*, 1985). If the nuclear extract contains proteins which specifically recognize and bind to the regulatory elements, the segment of DNA which is bound by protein is protected from DNase degradation. When the digested DNA is then run on a sequencing gel, it appears as a ladder of bands, each corresponding to a site of DNase cleavage, but a gap in the ladder appears where the DNA was protected from digestion by its protein factor. Using this type of assay, many of the sequences and proteins responsible for regulating gene expression have been identified. An alternate approach is to alter or delete sequences near the start of transcription and then assay the promoter activity, by re-introduction into cells or using an *in vitro* transcription system.

(i) *Promoter elements*

The promoter region can be loosely defined as the sequences necessary for correct initiation of transcription. By comparing many genes transcribed by polymerase II (which transcribes the genes for mRNA; Pol I transcribes rRNA genes and Pol III 5S RNA and tRNA genes), two conserved elements were found. These were the "TATA box" with the consensus sequence 'TATAAA', located 25 to 30 base pairs upstream of the initiation start (-25 to -30) (Corden *et al.*, 1980), and the CAAT box between -70 and -80 (Benoist *et al.*, 1980). By deleting these sequences and analyzing the efficiency and accuracy of transcription, it was established that the TATA box is necessary for initiation at the accurate start site (Grosveld *et al.*, 1981; Benoist and Chambon, 1981), whereas the CAAT sequence determines the efficiency of polymerase binding (McKnight *et al.*, 1984; Myers *et al.*, 1986; Bienz and Pelham, 1986).

TATA-binding factors have been identified in Drosophila and mammalian cell extracts (Parker and Topol, 1984; Davison *et al.*, 1983). Chromatographic fractionation and reconstitution of Drosophila nuclear extracts revealed that at least three components were required for initiation of transcription: RNA polymerase II and two other chromatographically distinguishable factors. One of these factors was partially purified and, using footprint analysis, found to bind and protect a 65 bp region of the histone H3 and H4 and actin 5C gene promoters (Parker and Topol, 1984). The protected region included the TATA box and was punctuated by 3 hypersensitive sites. Davison *et al.* (1983) used HeLa cell extracts, enriched for transcription factors by heparin-Ultrogel chromatography, to study specific binding to conalbumin and adenovirus promoters. They pre-incubated the protein extract with one of the DNA templates, then sequentially added the second DNA template, RNA polymerase and, finally, nucleotides to allow transcription to proceed, then analyzed the transcribed products by gel electrophoresis. In this way they demonstrated that factors present in the extract could form stable pre-initiation complexes with the template DNA, and it was this pre-initiation complex which RNA polymerase

recognized. Deletion of all or part of the TATA box region showed that it is involved in the stable formation of the pre-initiation complex. Fire *et al.* (1984) used a similar approach, but fractionated the HeLa cell extracts to establish the order of binding in more detail. At least two factors (two fractions separated by chromatography) bind the DNA first, then RNA Pol II, and finally, factor(s) in a third fraction. Together, this data suggests that the TATA region is required for sequential binding of transcription factors, which form a preinitiation complex necessary for RNA polymerase binding and initiation of transcription.

The CAAT-binding transcription factor (CTF) from HeLa cell extracts has now been purified to apparent homogeneity by affinity chromatography (Jones *et al.*, 1987). Using DNase footprinting, the binding site of CTF was localized to 18 to 23 bp around the CAAT regions of human alpha- and beta-globin, HSV1-tk , ras and hsp70 promoters (Jones *et al.*, 1985). Point mutations in the HSV-tk CAAT sequence eliminated CTF binding and decreased transcription to 10% of the wild-type level. The factor varies in its affinity for different CAAT sequences, depending on the extent to which the recognition sequence matches a consensus sequence, 'AGCCAA(T)' and whether the sequence is present in a 2-fold inverted repeat (Jones *et al.*, 1987). Binding affinity may also vary due to requirements for other synergistically-acting factors, depending on the promoter. For example, the hsv-tk CTF binding site is flanked on either side by an Sp1 (see below) binding site (Jones *et al.*, 1985), and the CAAT element of the mouse beta-globin promoter interacts with a beta globin-specific sequence, whereas the human alpha-globin CAAT element appears to function independently of other promoter elements (Dierks *et al.*, 1983; Charnay *et al.*, 1985, Myers *et al.*, 1986).

In addition to these two conserved sequences, other upstream promoter elements have been found which are more specialized. Like the CAAT box, these are involved in determining the efficiency of transcription. Some of these elements have been found in

many different promoters, whereas other elements are quite specialized (Dynan and Tjian, 1985). A few examples are presented below.

A factor which was originally thought to be required specifically for transcription of the SV40 promoters, but was subsequently found to activate transcription from several other viral and cellular promoters, is Sp1 (Dynan and Tjian, 1983; Dynan and Tjian, 1985). This factor recognizes a 'GGGCGG' repeating element, termed the 'GC box', although flanking regions modulate efficiency of binding to these sites. Sp1 is required for *in vitro* transcription of SV40 and the hsv-tk, monkey beta-region and mouse dihydrofolate reductase genes (Dynan and Tjian, 1985). In each case, multiple copies of GC boxes exist within 40 to 150 bp upstream of the RNA start site and are present in either orientation with respect to each other and the transcription start site. Potential Sp1 binding sites have also been found in the genes for human metallothionein (Richards *et al.*, 1984), adenosine deaminase (Valerio *et al.*, 1985), rat type II procollagen (Kohno *et al.*, 1985), mouse hypoxanthine phosphoribosyl transferase (Melton *et al.*, 1984) and other viral and cellular genes (Dynan and Tjian, 1985). The transcriptional control region of the retrovirus associated with AIDS has been shown to contain three Sp1 binding sites. Base substitution mutations of these sequences eliminated Sp1 binding (as detected by footprint analysis) and caused a 10-fold reduction of *in vitro* transcription activity.

Cells from a wide range of organisms (including Drosophila to man) have a conserved family of proteins which are rapidly synthesized in response to thermal stress. These are known as the heat shock proteins (hsp). Pelham (1982) first identified a conserved heat shock regulatory element (HRE) by transfecting COS cells with deletion mutants of the Drosophila hsp70 gene, or hsp70 gene promoter fused to the tk gene. In addition to the TATA box, a sequence at -48 to -62 was also required to confer heat shock inducibility. The element possesses dyad symmetry and appears to direct the efficient use of the TATA box in heat-shocked cells. At least 7 other hsp promoters have a sequence resembling, to a greater or lesser extent, the sequence in hsp70. DNase sensitivity

mapping of the hsp genes revealed that the 5' end of individual hsp 70 genes are nuclease hypersensitive, even before heat induction (Wu, 1980). Protein binding at the TATA box also preceded heat shock, as assayed by nuclease protection of chromosomal DNA within the 5' hypersensitive site. However, it was only after heat shock induction that proteins were bound at the TATA box and further upstream, both in Drosophila hsp70 and hsp83 gene promoters. This binding coincides with gene expression (Wu, 1984a). This lead the author to speculate that the binding of transcription factors at the TATA box, analogous to the pre-initiation complex with HeLa cell extracts, establishes the transcriptional potential of heat shock genes. The additional binding of heat shock activator protein (HAP) allows transcription to proceed. Finer mapping using cloned hsp70 and hsp82 DNA sequences (native DNA vs. chromatin) and nuclear extracts showed that the sequences protected upon heat shock induction corresponded to the elements identified by Pelham. In fact, the hsp82 gene, which is easier to induce and has higher affinity for HAP, has the consensus sequence as well as two similar elements overlapping it on either side (Wu, 1985). The heat shock elements of different hsp gene promoters compete for the same partially purified HAP factor, implying coordinate regulation of the gene family via binding of a common regulatory protein (Wu, 1984b).

Metallothionein (MT) genes encode heavy metal binding proteins and , in mammals, are induced independently by either heavy metal ions or glucocorticoid hormones (Mayo and Palmiter, 1981). Karin *et al.* (1984) used deletion mutants of the human MT-IIA gene, or a fusion of the MT-IIA gene promoter and the HSV-tk gene, transfected in rat fibroblasts to determine sequences necessary for metal responsiveness. They identified three types of regulatory elements: two copies of metal regulatory elements (MRE) at -38 to -50 and -138 to -150; one copy of a sequence necessary for basal level of expression at -70 to -90; and one glucocorticoid regulatory element at -237 to -268, which is also the glucocorticoid receptor binding site. They noted that the MRE is also found in another human MT gene, as well as a mouse and rat MT gene, and consists of a 12 bp

consensus sequence. Closer examination of the upstream regions of six different MT genes showed that each of them possessed between 5 and 8 copies of the MRE. The activity of each copy varies, but a minimum of two are required for significant induction of transcription by metal ions (Searle *et al.*, 1985). The clustering of these elements may be necessary for their specificity, since the MRE is small and statistically may occur frequently in the genome.

(ii) *Enhancers*

A class of elements which are not part of the promoter but have a significant effect on promoter efficiency are the enhancers. These must be located in cis with the promoter, but can be as far away as 1 kb, either upstream or downstream and in either orientation and still retain promoter-enhancing activity. They have a dramatic effect on the efficiency of transcription, increasing it up to 1000-fold, and can act on heterologous promoters (Voss *et al.*, 1986).

The immunoglobulin heavy and light chain gene enhancers were the first cellular enhancers to be identified (Calame, 1985). They were originally found by linking pieces of the immunoglobulin genes to SV40 early or beta-globin promoters. The DNA sequences which were able to direct efficient transcription of these enhancer-dependent promoters were then characterized. The sequences were able to enhance transcription independent of distance and orientation, but required a cis configuration relative to the promoter, thus fulfilling the definition of enhancers. Both the heavy and light chain gene enhancers are located in an intron between the joining and constant regions of their respective gene complexes (Benarji *et al.*, 1983; Mercola *et al.*, 1983; Bergman *et al.*, 1984; Picard and Schaffner, 1984). From this position they can enhance the V gene promoter most effectively when it is brought into the vicinity by gene rearrangement (Calame, 1985). Immunoglobulin genes require the enhancer to direct detectable transcription in transfected plasmacytoma cells; in other cell types, even the wild type gene is unable to direct a

detectable level of transcription. When linked to a heterologous promoter, the enhancers are still active only when used to transfect lymphoid cells (Picard and Schaffner, 1984; Benarji *et al.*, 1983; Gillies *et al.*, 1983). This suggests that the factor which interacts with the enhancer is tissue-specific, but such a factor has proved difficult to find. Initial *in vivo* DNA protection experiments indicated protein binding in the enhancer region occured only in lymphoid cells (Ephrussi *et al.*, 1985). However, *in vitro* experiments have failed to duplicate tissue specific protection. Nuclear extracts from B, T or Hela cells give the same pattern of DNase footprint (Weinberger *et al.*, 1986). One group suggests there may be slight differences in the pattern and that different proteins from each cell extract are recognizing DNA sequences in the same region (Augereau and Chambon, 1986). Until the nuclear factors from each cell type are purified and characterized, it will be difficult to draw conclusions from these experiments about the mechanism of tissue-specific enhancement. Since there are multiple sites of DNase protection, it is possible that there is more than one protein species binding the enhancer and the different combinations of these proteins determine the tissue-specificity of the enhancer. Baltimore has found that of four homologous elements in the heavy chain mu enhancer, three are recognized by different protein factors and do not compete with each other (Sen and Baltimore, 1986).

Enhancer sequences are also utilized by the steroid-regulated genes. Steroid hormones exert their regulatory activity by binding their cognate intracellular receptor, which alters the receptor such that the complex of receptor and hormone has high afffinity for specific DNA sites (Anderson,1984). An example of this type of gene regulation is the effect of glucocorticoids on the expression of mammary tumour virus (MTV) DNA in chronically infected cells (Ringold *et al.*, 1977). Transcription from the long terminal repeat (LTR) of MTV increases dramatically upon hormone stimulation via a glucocorticoid enhancer element. Binding assays, electron microscopy and nuclease footprinting of receptor bound to mouse MTV DNA showed that there are five regions of DNA binding, one upstream and four within the transcribed sequence (Payvar *et al.*, 1983). They are

bound independently and each with approximately the same affinity by the hormone receptor complex. DNase footprinting indicates there are multiple binding sites within each region. Some small consensus sequences (glucocorticoid response elements, GRE) were found within the bound segments (Scheidereit *et al.*, 1983; Payvar *et al.*, 1983) but they are loosely conserved. Overall, there was a surprising lack of homology between different binding sites (protected from DNase). The regions show homology to upstream regions of other genes induced by glucocorticoids, including the human metallotheinein IIA and the rat liver gene for tryptophan oxygenase, as well as a sequence postulated to be recognized by the progesterone receptor (Scheidereit *et al.*, 1983). A GRE has also been found in the first intron of the human growth hormone gene and was shown to confer glucocorticoid regulation (Slater *et al.*, 1986).

The upstream hormone-binding region was further characterized by fusing it and deletion mutants of it to the tk gene and promoter (Majors and Varmus, 1983). An element between 140 and 190 bp upstream of the mouse MTV transcription start was found to be sufficient to confer glucocorticoid responsiveness and can exert its activity independent of orientation and distance, thus seems to be an enhancer. Constructs of the MTV LTR (containing the upstream hormone-binding region) and the tk gene and promoter were used to produce stable L cell transfectants (Zaret and Yamamoto, 1984). Transcription from the tk gene promoter was dependent on the presence of hormone, and the entire region of the gene became 5- to 10-fold more sensitive to DNase approximately 20 minutes after hormone treatment. This overall DNase sensitivity was maintained after hormone withdrawal for at least 20 cell generations. They also found an induced hypersensitive site coinciding with the upstream DNA sequence that is bound by glucocorticoid receptor. This site took only 7 minutes to establish following hormone treatment, but was reversible upon hormone withdrawal, suggesting that the induced hypersensitive site forms only when the glucocorticoid-receptor complex is bound at the GRE (Zaret and Yamamoto, 1984).

Two lymphokines which are produced in helper T cells, gamma-interferon and IL2, may share an enhancer element. The genes for both are transcriptionally activated upon antigen and IL1 stimulation with approximately the same kinetics (Kronke *et al.*, 1985; Wiskocil *et al.*, 1985). The sequences which are required for the activation of the human IL2 gene were localized by fusing segments of the 5' flanking region and part of the first exon to the chloramphenicol acetyltransferase structural gene (CAT) and transfecting the helper T cell lines, Jurkat-111 and EL4 (Fujita *et. al.*, 1986). The cell lines were treated with TPA (12-O-tetradecanoyl phorbol-13-acetate) or ConA, which will induce IL2 expression, and then assayed for CAT activity. In this way, they found that the sequence between -319 and -264 from the transcription start allowed the transfected gene to be induced by mitogen stimulation. This segment was able to exert its transcriptional activation effect independent of orientation and was able to activate a heterologous promoter (the human beta-interferon gene promoter). This suggests the sequence is a regulatory enhancer, which is restricted to activated T cells, although it has not yet been shown to be effective over long distances from the promoter (Fujita *et al.*, 1986). Interestingly, the region which was found to be necessary to confer the specificity of IL2 expression overlaps a consensus sequence which was identified by homology to a gamma-interferon sequence. Potential regulatory sites of the human gamma-interferon gene were localized by assaying for DNase hypersensitive sites (Hardy *et al.*, 1985). A site was present in cells which can express the gene (Jurkat cells) but not in non-expressing cells. This hypersensitive site was present not only in mitogen-induced Jurkat cells, but also in unstimulated cells in which no gamma-interferon transcript is detectable. The site was mapped to a sequence in the first intron of the gene, and a computer search revealed a homologous sequence in the 5' flanking region of the human and mouse IL2 gene (21 matches of 24 bp). The data suggests that these genes, which are coordinately regulated, share a regulatory factor that recognizes a common element.

As may be apparent in the examples above, there is a fine line between "regulatory elements" and "enhancers". By definition, both potentiate the level of transcription from a promoter, but with varying degrees of distance and location independence and complexity. In some cases, a collection of regulatory elements, such as the HREs or MREs, might be considered an enhancer. A recurring theme as more is being learnt about the regulatory regions of genes is that more than one element is required and it is the combination of several regulatory DNA sequences which results in correct gene activation. Consequently, the current model holds that tissue-specific expression is conferred by a combination of regulatory elements and signal proteins which bind to them, not all of which are tissue-specific, and which may be either activators or repressors of expression (Voss *et al.*, 1986).

(c) *Post-transcriptional and Translational Controls*

An example of regulation via post-transcriptional processing, discussed previously, is the differential exon usage and splicing to generate membrane or secreted forms of immunoglobulin from a single gene. Another example is the tissue-specific expression of the calcitonin gene (Amara *et al.*, 1982). This gene produces a long primary transcript, identical in the thyroid and hypothalamus. However, by alternative processing of the primary transcript, two distinct mRNAs are produced. One encodes the hormone calcitonin, and is predominantly produced in the thyroid, and the other encodes calcitonin gene-related peptide (CGRP) and predominates in the hypothalamus.

Regulation at the level of translation can occur for almost all secreted proteins (Walter *et al.*, 1984 and references therein). Proteins destined for membrane insertion, secretion or a cytoplasmic organelle possess N-terminal signal sequences which are recognized and bound by signal recognition particle (SRP), an 11S cytoplasmic ribonucleoprotein (Walter and Blobel, 1983). This recognition occurs shortly after the initiation of translation, when the signal sequence protrudes from the ribosome, and

effectively blocks the progression of translation (Walter and Blobel, 1981). The arrest of translation is released when the SRP-translation complex binds to the SRP receptor (or docking protein) on the endoplasmic reticulum (ER) membrane. The protein is then translated and translocated through the membrane of the ER, with concomitant cleavage of the signal sequence, from where it will be segregated to its correct destination. This control may be to ensure that proteins destined for the cell surface, cytoplasmic organelles or exterior are not synthesized in the cytoplasm, but are directed to the ER. It also provides the cell with another mechanism for a rapid and regulatable response to external stimuli (Walter *et al.*, 1984).

The immunoglobulin light chains were among the first proteins with which this mechanism was discovered and investigated. Messenger RNA from murine myelomas, which express high levels of immunoglobulins, was used to demonstrate that the information for the segregation of a translation product is in the mRNA, not in the translation machinery (Blobel and Dobberstein, 1975). The lymphokines, all of which are secreted, also possess signal sequences, an important realization for *in vitro* translation studies, since translation will not normally proceed in rabbit reticulocyte systems without the exogenous addition of docking protein (Harnish *et al.*, 1986). A curious exception is IL1. The gene for IL1 has been cloned and from the cDNA sequence this lymphokine does not appear to have a signal sequence, yet is released from the cell (Lomedico *et al.*, 1984; March *et al.*, 1985; Auron *et al.*, 1984). It will be interesting to see how secretion of this protein occurs.

(d) *Stability of mRNA*

Finally, expression may also be regulated by altering the stability of the mRNA. A wide variation exists in the half-life of different mRNAs. The transcripts for transiently expressed proteins such as c-fos or c-myc have half-lives as short as 15 minutes, whereas other transcripts, such as that for beta-globin, are very stable (Brawerman, 1987). Certain

structural features of mRNA have been identified which influence their resistance to degradation, however relatively little is known about this mechanism of regulation of expression. The poly(A)-tail of mRNA is one general feature which seems to confer some stability, since removal of it leads to rapid degradation (Huez *et al.*, 1981). In some oncogenes and lymphokines, specific sequences in the 3' end which shorten the mRNA half-life have also been found. Experiments using hybrid constructs of GM-CSF and beta-globin sequences suggested that an AT-rich sequence in the 3' untranslated region of GM-CSF mRNA may target it for rapid turnover (Shaw and Kamen, 1986). Several lymphokines and inflammatory mediators, as well as protooncogenes, were subsequently found to possess a potential consensus sequence in the 3'-untranslated region of their mRNAs, contained in the AT-rich region. Several of these short-lived mRNAs accumulate rapidly when cellular protein synthesis is inhibited. An important feature of lymphokine production is its rapid increase and decline in expression. This pattern of expression helps to restrict the immune response to the location and continued presence of antigen. If the 3' consensus sequence targets the lymphokine mRNAs for rapid degradation, the observed burst of expression is due at least in part to regulation of the mRNA half-life.

2. *Recombinant DNA Methods*

The advent of recombinant DNA technology has provided a powerful tool with which to solve traditional biochemical problems. By inserting a piece of foreign DNA into a plasmid or phage vector and propagating this recombinant DNA molecule in bacteria, clones carrying the identical DNA information can be produced. This allows the study of DNA sequences or their corresponding protein products in isolation from other sequences, and permits the production of large amounts of material to study. These techniques are briefly described in this section.

(a) *Construction of cDNA Libraries*

Complementary DNA libraries represent the mRNA population of a cell, therefore only genes which are expressed are included. As starting material for the library, messenger RNA is isolated from a cell line or tissue of interest. A complementary DNA strand (cDNA) to the RNA is synthesized by priming with oligo-d(T), which hybridizes to the 3' poly(A) tail of the mRNA, and extending the primer using the enzyme reverse transcriptase. The cDNA is made double stranded in one of several possible ways. The traditional method was to hydrolyze the RNA template with NaOH, then synthesize the second strand of DNA using DNA polymerase primed from transiently-formed hairpin structures of the cDNA (Maniatis *et al.*, 1982). This method is relatively inefficient, since the hairpin structures exist transiently and cloning artifacts can be generated if too much secondary structure exists. An alternate method is to incubate the DNA-RNA hybrid with RNase H, T4 DNA polymerase and T4 DNA ligase (Gubler and Hoffman, 1983). The RNase H introduces nicks in the RNA, from which T4 DNA polymerase can prime and synthesize a second strand of DNA, removing the RNA via its powerful exonuclease activity. T4 DNA ligase then joins the newly synthesized DNA fragments. This method is not only more efficient than the previous one, but it favors the synthesis of full-length clones. A third approach is to tail the 3' end of the cDNA strand with deoxyguanosines (G's) (Land *et al.*, 1981). After digestion of the RNA template with RNase A, oligo-d(C) is added to hybridize with the oligo-d(G) tail. This provides the primer from which DNA polymerase is then used to synthesize the second strand of DNA. Although this method is efficient, information at the ends of the mRNA may be lost due to incomplete elongation from the oligo-d(T) or oligo-d(C) primers.

The double-stranded complementary DNA (dscDNA) must then be modified for insertion into a bacterial vector. The most common method now used is to ligate 'linkers' to the dscDNA ends, using T4 DNA ligase (Goodman and MacDonald, 1979). These linkers are short double-stranded pieces of DNA which contain the recognition site of a

restriction enzyme, and when cleaved with that enzyme, will produce sticky ends (short single-stranded protruding ends). The dscDNA can then be ligated into a vector which has been cut with the same restriction enzyme and thus has the complementary sticky ends. The vector is replicated and carried by host bacterial cells, so it serves as the vehicle for the foreign DNA. Two types of vector used are plasmid vectors and bacteriophage vectors (Maniatis *et al.*, 1982). Plasmid vectors are relatively small (normally <10 kb) and are used to transfect bacterial cells directly, by incubating the DNA with the cells in the presence of $CaCl_2$. Bacteriophage DNA is packaged *in vitro* into infectious virus particles before transfecting the bacterial host. This represents a much more efficient method of introducing the recombinant DNA into the host cells, so that more clones are generated per mole of dscDNA. However, once a clone of interest is identified, it usually must be subcloned into a plasmid vector, since phage DNA is quite large, typically 30 to 60 kb, which makes it difficult to work with the insert DNA, usually only 1 or 2 kb. The vector, whether phage or plasmid, normally carries a selectable marker, so that cells carrying recombinant DNA can be selected for. Regardless of the vector used, the population of cells which has been transformed with the recombinant DNA is referred to as a cDNA library.

(b) *Screening cDNA libraries*

Most often, the purpose of screening a cDNA library is to isolate a clone representing a previously characterized protein. In this case, one possibility is to express the protein in its bacterial environment and screen using antibodies to the protein or a functional assay (Huynh *et al.*, 1985). A more common approach, if some of the protein sequence is known, is to synthesize and radioactively label the corresponding DNA sequence to use as a probe to screen the library (Moriuchi *et al.*, 1983).

Another approach is to isolate clones which are specifically expressed in the cells from which the library was generated, without knowing what proteins they would encode.

The rationale behind this approach is that every cell must express, in addition to common genes required for 'house-keeping' duties, a characteristic set of genes necessary for that cell's specialized function. By identifying such cell-specific genes. and determining the nature of the protein product, information can be gained regarding how that type of cell carries out its function (Crampton *et al.*, 1980; Mather *et al.*, 1981; Milner and Sutcliffe, 1983).

Two general methods for identifying cell-specific clones are differential and subtractive screening. Differential screening is carried out by synthesizing radioactive cDNA from several cell types to use as probe . The types of cells should include the cells of interest and other related cells which do not carry out the same functons. When the library is screened with the probe generated from the cells of interest, every clone should hybridize, since all messenger RNA species expressed in that cell are represented both in the library and in the probe. However, when the cDNA library is screened with probe from a related cell type, only clones which represent mRNAs common to both cell types will hybridize with the probe and give a positive radioactive signal. Clones which were derived from cell-specific mRNAs are not represented in probe made from other cells and give no signal or a weak signal. It is these clones which are of interest. Examples of clones which were identified by this technique are PDGF-inducible sequences (Cochran *et al.*, 1983) and adipose genes which are developmentally regulated (Ringold *et al.*, 1986).

The method of subtractive screening is similar, but rather than successively probing the library with cDNA made from different types of cells, a large amount of high specific activity probe is synthesized from the cell type of interest. This probe is then hybridized successively with mRNA from other types of cells. Common mRNAs hybridize to their complementary DNA in the probe, "subtracting" them out. The remaining single-stranded material represents cell-specific mRNAs and can be purified by HAP-chromatography (Davis *et al.*, 1982). After several rounds of subtraction of common sequences, the

remaining cell-specific probe is used to screen the cDNA library, and clones which are hybridized by the probe are the cell-specific clones of interest.

The method of subtractive screening is a much more sensitive one, since the criteria is a positively hybridizing clone, whereas in differential screening, clones which are relatively more positive with one probe must be identified. For low abundance mRNAs, the difference in signal intensity between probe derived from expressing and non-expressing cells may not be significant, consequently subtractive screening is the method of choice if clones of low abundance are desired. In fact, this method was originally designed in order to isolate a clone for the elusive T cell receptor (Hedrick *et al.*, 1984). However, to use subtractive screening, a large amount of mRNA from the cells of interest must be available as starting material for synthesis of the probe, since only a small fraction (approximately 0.3%) is left after subtraction and at least two rounds of screening must be carried out to eliminate background positives.

(c) *Genomic Clones*

A cDNA library represents the mRNA species in a particular type of cell and therefore doesn't contain any information on the gene structure or how the transcription of the gene is regulated. For this sort of information, a genomic clone must be isolated. Genomic libraries are constructed by shearing or partially digesting genomic DNA with a restriction enzyme, to generate an average length of 20 kb (Maniatis *et al.*, 1982). This is then ligated to a bacteriophage vector and packaged for transfection of a host bacteria. These large genomic DNA clones are used to determine the exon/intron arrangement of the genes and map the promoter region and possible regulatory sequences.

C. FOCUS OF THIS WORK

The aim of this study has been to use the power of molecular genetic techniques to study (1) how a cytotoxic T cell carries out its function and (2) control of expression of CTL-specific genes.

Although B and T_H cells have been the focus of intense research in the past, CTL remained neglected, due in large part to the lack of available cell lines until recent years. Therefore the mechanisms by which a killer T cell lyses its target have remained quite mysterious. Assuming CTL possess a characteristic set of proteins to carry out their specialized function, these must be encoded by mRNA transcripts which are CTL-specific. My approach to identifying molecules which are necessary for killer cell function has been to generate a cDNA library from a cloned cytotoxic T cell line and use differential hybridization analysis to identify recombinants which represent mRNAs transcribed in mouse cytotoxic T cells and not in other cell types.

The identification and characterization of cDNA clones isolated in this manner are discussed in the following chapter. The sequence and implicated function of the protein products is then presented. The next chapter describes the isolation and mapping of the corresponding genomic clones, and localization of regions of the genes which may be involved in regulation.

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CHAPTER II

CLONING OF TWO GENES THAT ARE SPECIFICALLY EXPRESSED IN ACTIVATED CYTOTOXIC T LYMPHOCYTES[1]

A. INTRODUCTION

Thymus-derived (T) cells play a major role in the immune system. A number of T lymphocyte subsets can be distinguished whose functions have been categorized as regulatory, cooperative, or effector. These subsets regulate immune responses by either suppression or supplying help, are necessary for the induction of B-cell responses, and are capable of lysing cells bearing foreign antigens. However, the genesis of these diversely programmed lymphocytes appears to be from a single pluripotent stem cell (Wu *et al.*, 1968). Recent developments in T cell tumor biology and cloning have made both functional end stage cells (Moller, 1981) and precursor cells (Nabel *et al.*, 1981) available for biochemical and genetic analysis. Many surface components of functional T cells and their precursors have been serologically characterized (McKenzie and Potter, 1979) and their genes mapped. Studies on the changes in these cell-surface antigens as cells pass from the bone marrow through the thymus to peripheral tissues have been very rewarding in understanding the complex process of T cell ontogeny (Cantor and Boyse, 1977). However, the basic problem of this type of approach is that it gives no information about the molecular or genetic basis of the phenotypic changes. Recently, the power of molecular genetic technology has been used to clone the genes for a number of these serologically defined T cell antigens, including Thy-1 (Moriuchi *et al.*, 1983), Lyt-2,3 and Leu-2/T8

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(Kavathas *et al.*, 1984), T3 (van den Elsen *et al.*, 1984), and Tac (Leonard *et al.*, 1984). These cloned genes can now be used as probes to address many of the intriguing questions suggested by serology. Already the cloning of the T cell antigen receptor genes (Hedrick *et al.*, 1984; Yanagi *et al.*, 1984) has led to a tremendous increase in understanding how T cells recognize antigens.

Most serological and genetic studies on T cells have concentrated on molecules that are expressed on the cell surface. The internal mechanisms by which the different T cell subsets carry out their specific functions remain largely unknown. However, as the phenotype of a cell is governed by the proteins expressed, it may be assumed that each subset of cells must express a characteristic set of function-related proteins and the corresponding mRNAs. Thus, a general approach to identify and isolate mRNAs that are expressed in the cells of interest but at very low levels or not at all in other types of cells, should provide vital information on the nature of the molecules involved in cell-specific functioning. This type of approach has been extremely rewarding in a number of different systems (Gray and Goeddel, 1982; Hastings and Emerson, 1982; Milner and Sutcliffe, 1983; Milner *et al.*, 1984; Hirschhorn *et al.*, 1984; Kelly *et al.*, 1983). By differential hybridization analysis, we have identified cDNA clones that are expressed in restricted T-cell subsets. In this chapter the characterization of two related cDNA clones that are expressed in murine cytotoxic T cells and not in helper lines or thymocytes is described. The expression of the two mRNAs encoded correlates with the induction of cytotoxicity, thus suggesting that they may encode proteins that are vitally important in killer cell activation or may be involved in the lytic mechanism itself. Undoubtedly these clones will provide very useful markers to study the molecular events involved in the regulation of gene expression during T cell ontogeny. Moreover, identification of the proteins they encode may provide clues to how these specialized end-stage cells function in target-cell lysis (Berke, 1983).

B. MATERIALS AND METHODS

1. *Cells*

The cytotoxic T cell lines MTL2.8.2 and MTL11.1 were generated from CBA/J mice as described (Bleackley *et al.*, 1982). EL4.E1 is an interleukin 2 (IL2)-producing variant of the EL4 cell line (Farrar *et al.*, 1980). CH1 is a CBA/J anti-(CBA/J x BALB/c) antigen-specific helper T cell line. It was produced from a 2-day mixed lymphocyte culture by continuous restimulation with irradiated F1 spleen cells in RPMI 1640 medium supplemented with 10% fetal bovine serum and 100 uM 2-mercaptoethanol (RHFM). To generate human cytotoxic T lymphocytes (CTL), peripheral blood lymphocytes were incubated in RHFM and stimulated with irradiated allogeneic cells at days 0 and 7 and harvested at day 10. Fetal-derived cells were kindly provided by H.-S. Teh (UBC; Teh *et al.*, 1985).

For the time course of cell activation, spleen cells from CBA/J mice were incubated in RHFM (10^6 cells per ml) and purified IL2 (Riendeau *et al.*, 1983), either with an equal number of mitomycin C-treated EL4.E1 cells or Con A (2 ug/ml). Samples were removed at day 1 through day 6, assayed for cytotoxic activity (Shaw *et al.* , 1980) and analyzed by cytodot hybridization.

2. *cDNA Library Construction*

Double-stranded cDNA was synthesized from 4 ug of MTL2.8.2 mRNA (Bleackley *et al.*, 1982; Chirgwin *et al.*, 1979) as described by Gubler and Hoffman (1983). Following repair with the Klenow fragment of E. coli DNA polymerase and T4 DNA polymerase to maximize flush ends, phosphorylated EcoRI linkers (P-L Biochemicals) were ligated to the cDNA in 70 mM Tris-HCl, pH 7.6/10 mM $MgCl_2$/5 mM dithiothreitol/1 mM ATP/1 unit of T4 DNA ligase at 14 C overnight (Goodman and MacDonald, 1979). After digestion with EcoRI the product was run on a 5 ml Sepharose

4B column, and the excluded fractions were pooled and ethanol-precipitated. The cDNA was ligated to EcoRI/bacterial alkaline phosphatase-treated pUC13 (P-L Biochemicals) in 66 mM Tris-HCl, pH 7.6/6.6 mM $MgCl_2$/10 mM dithiothreitol/1 mM ATP. Reactions were heated to 37 C for 5 min, quick-chilled before the addition of 1 unit of T4 DNA ligase, and incubated at 14 C for 2 hr. Escherichia coli JM83 cells were made competent by using the $CaCl_2$/RbCl procedure described by Maniatis *et al.* (1982) and were transformed with the ligated cDNA. White colonies (those containing inserts) were ordered in 96-well microtiter plates and stored in LB medium containing 20% glycerol at -70 C.

3. *Differential Screening*

Colonies were replicated in triplicate onto nitrocellulose filters (Schleicher & Schuell), grown for 6 hr, and then amplified on chloramphenicol (100 ug/ml) for 12 hr. Bacteria were lysed and the filters were baked and prewashed to remove bacterial debris (Maniatis *et al.*, 1982). Prehybridization at 42 C for 12-20 hr was done in 50% (vol/vol) formamide containing 2XDenhardt's solution (1XDenhardt's solution = 0.02% polyvinylpyrrolidone/0.02% Ficoll/0.02% bovine serum albumin), 4XSET buffer (1XSET buffer = 0.6 M NaCl/0.12 M Tris-HCl, pH 8/1 mM EDTA), 0.1% NaDodSO4, 100 ug of yeast tRNA per ml, and 125 ug of poly(A) per ml (Sigma). Hybridization in the same buffer included 1 to 5 x 10^5 cpm of cDNA probe per ml synthesized from mRNA with 20 ug of T-primers per ml (Collaborative Research, Waltham, MA); 50 mM Tris-HCl (pH 8.3); 10 mM $MgCl_2$; 5 mM dithiothreitol, 500 uM each of dGTP, dATP, and dTTP; 70 mM KCl; 30 uCi (1 Ci = 37 GBq) of [alpha-^{32}P]dCTP (New England Nuclear, 3000 Ci/mmol); and 15 units of avian myeloblastosis virus reverse transcriptase at 42 C for 60 min. Template RNA was hydrolyzed by the addition of NaOH to 1.5 M. Samples were boiled for 3 min and fractionated by Sephadex G-50 column chromatography. Filters were washed in 5XSET buffer for 15 min at 22 C and then in 2XSET buffer/50% formamide for 20 min at 42 C and were exposed to film (Kodak X-Omat AR) with an

intensifying screen for 1 to 3 days at -70 C. Hybridized probe was removed by boiling the filters for 10 min in distilled water.

4. *Blot Analysis*

Cytodots were prepared as described by White and Bancroft (1982). For blot-hybridization analysis, total cytoplasmic RNA (10 ug) or poly(A)$^+$ mRNA (2 ug) was denatured in 6.3% formaldehyde/50% formamide at 55 C and size-fractionated on a 0.8% agarose gel containing 0.66% formaldehyde. RNA was transferred to nitrocellulose as described by Thomas (1980). Plasmid DNA was digested with EcoRI, run on a 0.7% agarose gel, and transferred to nitrocellulose (Southern, 1975). Filters were baked at 80 C for 2 hr, then prehybridized at 42 C for 6-12 hr in 50% formamide containing 20 mM phosphate buffer (pH 6.8), 2 mM pyrophosphate, 100 µM ATP, 5x Denhardt's solution, 0.75 M NaCl, 0.075 M sodium citrate (pH 7), 100 µg of salmon sperm DNA per ml, 0.1% NaDodSO4, 50 µg of poly(A) per ml, and 2.5 mM EDTA. Hybridization was carried out in the same buffer with a nick-translated plasmid of specific activity 1 x 10^8 cpm/µg (Bethesda Research Laboratories kit) at 1 x 10^6 cpm/ml.

C. RESULTS

1. *cDNA Library and Differential Screening*

Triplicate copies of the library were hybridized first with cDNA synthesized from MTL2.8.2 mRNA (Bleackley *et al.*, 1982), then, after autoradiography and washing, with helper T cell cDNA, and finally with thymocyte cDNA . Colonies that gave a higher hybridization signal with killer cell mRNA in at least two of the three copies of the library were picked. Upon rescreening, again in triplicate, 36 of these 121 colonies appeared to be clearly CTL-specific. Plasmid DNA isolated from these colonies was cut with EcoRI, and a series of cross-hybridizations was performed. Two clones were chosen for more extensive analysis: clone B10 because it appeared to be the most abundant in the library,

cross-hybridizing strongly with eight other inserts, and clone C11 because it weakly cross-hybridized with B10 but not with all B10-related clones (one other C11-related sequence was found). The inserts of clones B10 and C11 have been sequenced and are shown in figure 1.

2. *B10 and C11 Are Killer Cell Specific*

Cytodots (White and Bancroft, 1982) prepared from a variety of cells and tissues were hybridized with nick-translated B10 and C11. Results are shown for B10 (figure 2A). The data with probe C11 were similar and are not shown. The highest signal was detected in MTL2.8.2--i.e., the killer cell line that was used to generate the cDNA library. A weaker but positive signal was observed with MTLIII, a variant of MTL2.8.2 that had a low level of cytotoxicity and had become IL2 and antigen independent (J. Hooton, personal communication). A similar level of expression was observed in a novel T cell clone derived from murine fetal thymus (Teh *et al.*, 1985). There was no evidence for expression in either mouse thymocytes or a helper T cell line (CH1) that secretes IL2 in response to antigen. Mouse brain and liver and a human CTL line were similarly negative under the high-stringency conditions of this experiment. In addition to the data given in figure 2, no evidence for expression of B10 or C11 was found in either a sample of enriched natural killer cells (Kumagai *et al.*, 1982), even after interferon stimulation (Djeu, 1979), or a helper T cell hybridoma that secretes an antigen-specific factor (Kwong *et al.*, 1984). To ensure that the negative samples did contain hybridizable RNA, all of the cytodots were reprobed with either a lymphocyte-specific probe (unpublished data), or oligo(dT) or the T-cell antigen receptor beta-chain gene (Hedrick *et al.*, 1984). Although the level of signal varied, all samples were positive.

To enrich for the B cells of a spleen cell suspension, lymphocytes were separated from adherent cells on Petri dishes and then treated with anti-Thy-1,2 antiserum. The enriched B cells were then incubated with lipopolysaccharide (LPS) or Con A or medium.

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B10   AAAACACTGCACGAAGTTAAGCTGACAGTACAGAAGGATCAGGTGTGTGA

B10   GTCCCAGTTCCAAAGTTTTTACAACAGAGCTAATGAGATATGTGTGGGAG

B10   ACTCAAAGATCAAGGGAGCTTCCTTTGAGGAGGATTCTGGAGGCCCGCTT

B10   GTGTGTAAAAGAGCAGCTGCAGGCATCGTCTCCTACGGGCAAACTGATGG
                              ***** ** ***** **  * *  *****
C11                           GGCATAGTTTCCTATGGATATAAGGATGG

B10   ATCAGCTCCGCAAGTCTTCACAAGAGTTTTGAGTTTTGTATCGTGGATAA
       *** **** *  *  ***** * *** * ******  **** ********
C11   AGAAAACAATGAAAAGCAGCTAACTAC AGAAGCAACATGGATCCTGCTC

B10   AGAAAACGATGAAACACAGCTAACTACAAGAAGCAAC TAGATCCTG AC
      ******** ******  ********** ********* * *******  *
C11   AGAAAACAATGAAAAGCAGCTAACTAC AGAAGCAACATGGATCCTGCTC

B10   TGA CAGCCATCTTCCC ATAGCTGAGTCCAGGATTGCTCTAGGACAGAT
      ***  * ***** **** * ****************************** *
C11   TGATTACCCATCGTCCCTAGAGCTGAGTCCAGGATTGCTCTAGGACAGGT

B10   GGCAGGCAACTGAATAAAGAACTTTCTCTGACTGCAAAAAAAAAA
      ****** * ********** ** * *   *****
C11   GGCAGG ATCTGAATAAAGGAC TGCAAAGACTGGCTTCATGTCCATTCA

C11   CAAGGACCAGCTCTGTCCTTGGCAGGCCAATGGAACACCTCTTCTGCCAC

C11   CATGCTGTGACAACCCAACTGACATCTTCCTATGGAAGTTTGCCCTCTCC

C11   ACAAAAGAAGTAGAATGTTTGCATTGGAGCTGGGCATGCTCTGCTTCCCC

C11   TCAGTGCCCCGAGAATGTTATCTAATGCTAGTCATCATTAATAGCTCCCT

C11   ACAGAACTTTCATACAGTTGCACCCAAGTTGCTGATGTGTTCTCTAGAAT

C11   AGAGCAAGAAATAGTAAACA
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FIGURE II.1 Nucleotide sequence comparison of B10 and C11 inserts. The inserts of B10 and C11 were recloned in M13 and sequenced by the dideoxy method of Sanger *et al.* (1975). The sequences have been optimally aligned with asterisks designating corresponding nucleotide identities.

FIGURE II.2 Tissue specificity of expression. Cytoplasmic dot blots were hybridized with nick-translated B10 (A and B) or a mu heavy chain probe (C). The number of cells X 10^{-4} is shown. (B and C) Spleen cells were enriched for B lymphocytes by nonadherence to Petri Dishes and anti-Thy-1.1 treatment (time zero) and were incubated with LPS, ConA or RHFM medium alone, for 24 hr.

After 24 hr, the cells were harvested, cytodots were prepared, and the filter was probed with B10 or C11 (figure 2B). No expression of either sequence could be detected in any sample. However, when the blot was hybridized with an immunoglobulin μ heavy chain probe (Calame *et al.*, 1980), a strong positive signal was seen in the LPS-stimulated cells (figure 2C).

3. *Blot-Hybridization Analysis with B10 and C11*

Poly(A)$^+$ RNA was isolated from a variety of cell sources, run on a denaturing agarose gel, and transferred to nitrocellulose. Figure 3 shows the same filter probed first with nick-translated B10 (A), then with C11 (B), and finally with probe 10 (C), a cloned gene that detects mRNA in a variety of cell types (Paetkau *et al.*, 1984). Probe B10 detected a single band (~1000 bases) in two different murine cytotoxic T cell clones, MTL2.8.2 (figure 3, lane 2) and MTL11.1 (figure 3, lane 3). No bands were detected in RNA from thymocytes (figure 3, lane 1), an antigen-specific helper cell line (figure 3, lane 4), or murine thymoma EL4 (figure 3, lane 5). When the blot was reprobed with C11, again only the two cytotoxic T cell clones showed bands. However, in contrast to B10, this probe hybridized to two bands, one of ~1000 bases and the other of ~1400 bases. In addition to the results shown in figure 2, blot-hybridization analysis was performed on poly(A)$^+$ RNA from a number of murine cells including five CTL lines, brain, liver, three helper T cell lines, unstimulated and LPS-stimulated B lymphocytes, and one B cell myeloma. Of these, only the actively cytotoxic T cells expressed mRNAs that hybridized with B10 and C11. To ensure that all tracks contained hybridizable RNA, the blot was rehybridized with probe 10. A band of the expected size was seen in all tracks.

4. *B10 and C11 Are Induced During CTL Activation*

CBA/J (H-2^k) spleen cells were stimulated with either mitomycin C-treated EL4 cells or Con A. On each of the 6 days after stimulation, the level of cytotoxicity was

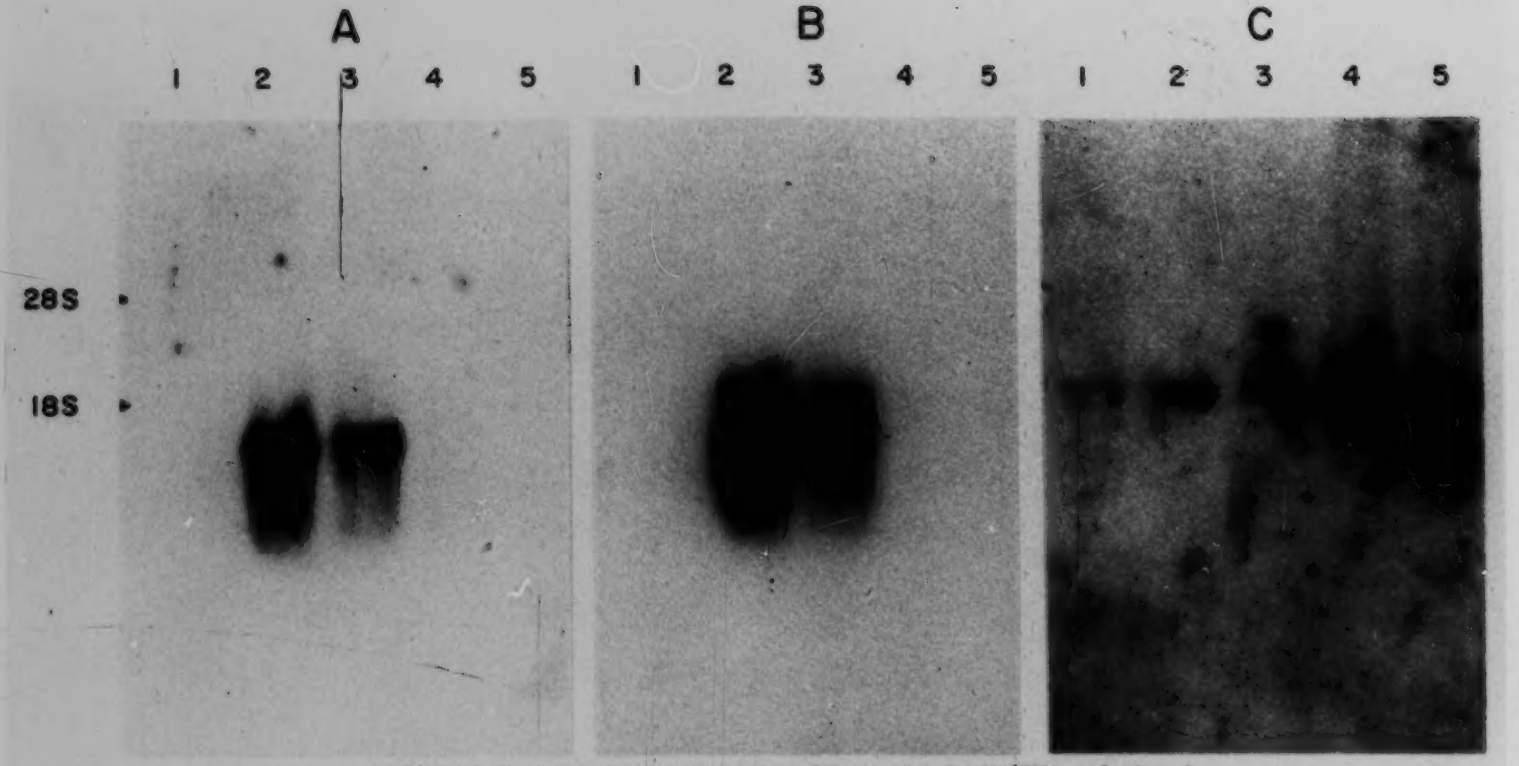

FIGURE II.3 Blot-hybridization analysis of B10 and C11 expression. Poly(A)$^+$RNA was fractionated on a formaldehyde-agarose gel, transferred to nitrocellulose, and hybridized with probes B10 (A), C11 (B), and 10 (C), a clone expressed in all cell samples. Samples of mRNA were prepared from thymus (lane 1); from MTL2.8.2 (lane 2) and MTL11.1 (lane 3), two different murine CTL lines; from an antigen-specific helper T-cell line (lane 4); and from EL4, a murine thymoma that secretes IL2 (lane 5).

measured in a chromium-release assay (Shaw *et al.*, 1978) against EL4 (H-2^b), S194 (H-2^d), and RI (H-2^k). Cytodots were also prepared on each of these days, and the blots were hybridized with nick-translated B10 and C11. Again, data are presented only for B10 (figure 4), as C11 gave indistinguishable results. Relative mRNA levels were determined by scanning densitometry on an ELISA plate reader. In the allo-specific response, the peak of cytotoxicity was observed on day 4, while the peak of B10 or C11 mRNA expression appeared to be on days 3 and 4. The peak of killing activity in the Con A-stimulated cells was also at day 4; however, the peak of mRNA expression was very sharply on day 3. In both experiments, the mRNA expression was reduced to background levels by day 6, while there were still significant levels of cytotoxicity on this day. When the cytodots were hybridized with ^{32}P-end-labeled oligo(dT), the peak of total mRNA was seen on day 2 (data not shown).

D. DISCUSSION

The approach used in this work -- i.e., studying cell-specific functions by mRNA population analysis -- is based upon the hypothesis of Hastie and Bishop (1976). They postulated that a small set of cell specific, abundant mRNAs were vitally important in the end-stage functions of a cell. In order to identify such mRNAs in cytotoxic T cells, a cDNA library of 4000 recombinants from a murine cytotoxic T cell clone (MTL2.8.2) was screened by differential hybridization by using cDNA probes synthesized from the mRNA of the same cytotoxic T cell clone, an antigen-specific helper T cell clones (CH1), and mouse thymocytes. All screening was performed in triplicate to minimize false positives caused by the technical difficulties of colony transfer. After rescreening potentially interesting clones, 36 clearly contained sequences that were expressed at reasonably high levels in cytotoxic cells and not at all, or at very low levels, in the other two cell types. The helper cell clone represents an alternative pathway of T cell differentiation to a functionally distinct cell type, while thymocytes represent a heterogeneous population of precursors

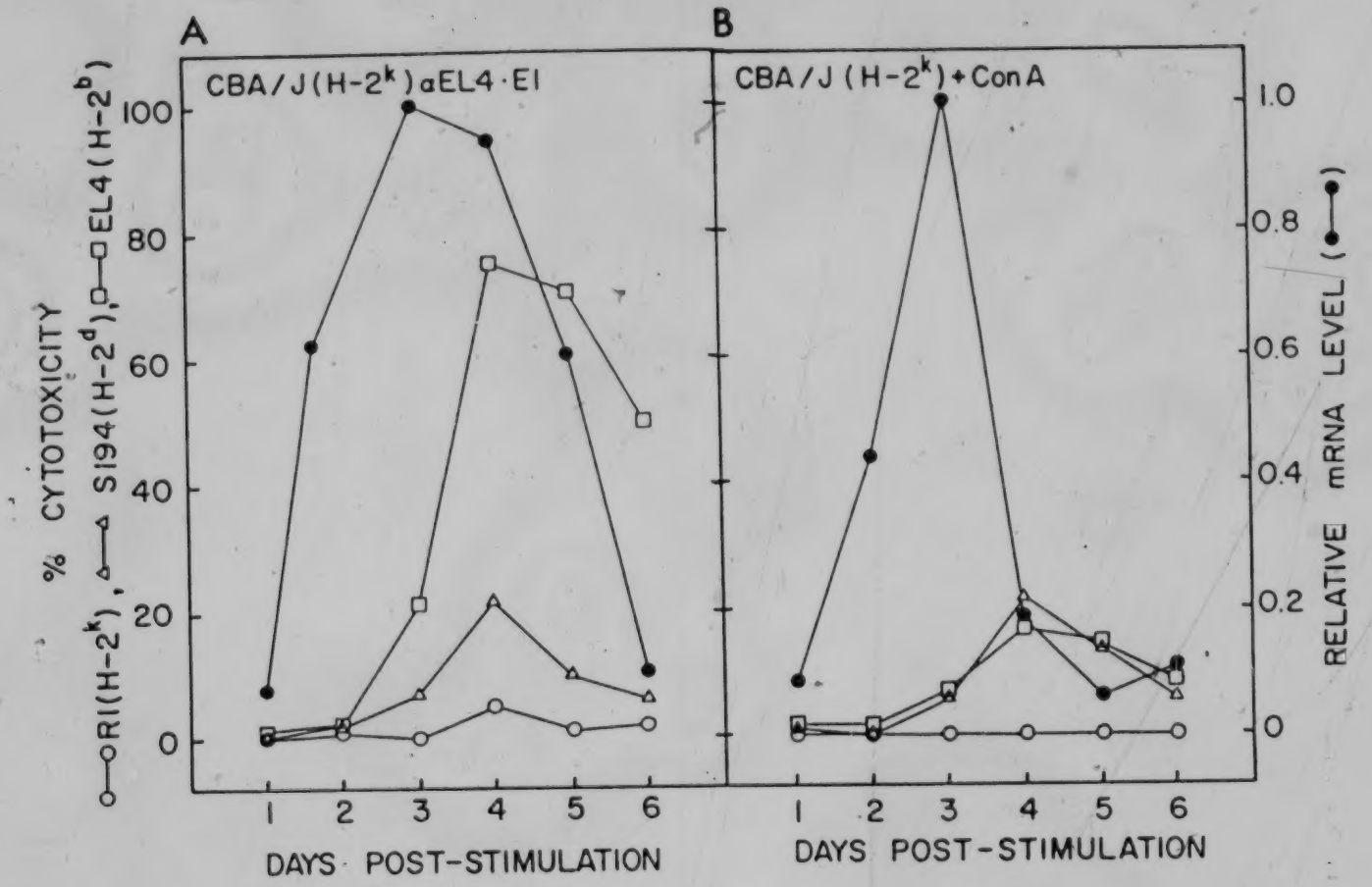

FIGURE II.4 Correlation of B10 mRNA expression with cell activation in a mixed lymphocyte culture. Spleen cells from CBA/J (H-2k) mice were stimulated with mitomycin C-treated EL4.E1 cells (A) or ConA (B) and were analyzed for cytotoxic activity.

including precursor cytotoxic cells (Cantor and Weissman, 1976). By screening with both thymocyte and helper cell cDNA, we hoped to identify clones that were expressed during cytotoxic lymphocyte activation. These cloned DNA sequences should include those encoding proteins directly involved in the lytic mechanism.

Two clones were selected for more extensive analysis. Clone B10 was chosen because it appeared to represent a highly abundant mRNA expressd in cytotoxic T cells, and clone C11 was chosen because cross-hybridization analysis indicated some partial homology between it and B10. To establish that these clones were indeed CTL-specific, cytodots were prepared from a number of sources and hybridized with the two cloned sequences (figure 2). The only cell type, other than CTL, in which expression of B10 or C11 was detected is a novel T cell isolated from mouse fetal thymus. Its relationship to other T cell subsets remains to be determined. Neither sequence was expressed in thymocytes, helper T cells, B lymphocytes, activated natural killer cells, mouse brain cells, and mouse liver cells. At the high stringency of washing used in these experiments, no cross-hybridizing material was seen in human CTL; however, a low level of homology cannot be ruled out. To exclude the possibility that B10 and C11 were expressed generally in activated lymphocytes, an enriched population of B lymphocytes from spleen cells was stimulated with LPS (B-cell mitogen). This activation did not result in expression of either of the CTL-specific sequences but did stimulate a massive increase in the level of μ heavy chain mRNA in these cells.

Blot-hybridization analysis (figure 3) with these two probes on poly(A)$^+$ mRNA from a number of cell sources confirmed the tissue-specificity results of the cytodot data. However clone B10 hybridized to a single mRNA species of ~1000 bases whereas C11 hybridized to two mRNAs of ~1000 and ~1400 bases. Sequence analysis of the inserts of B10 and C11 indicates that they are encoded by different genes, although over a stretch of 211 bases they are 80% homologous (figure 1), suggesting that they are part of a related gene family. The C11 transcript and B10 transcript probably correspond to the 1400 ntd

and 1000 ntd bands respectively, since they consistently hybridized to those bands. The apparent cross-hybridizatioon due to the homology between the two sequences is frequently seen, dependent upon the probe concentration in the hybridization and the length of exposure of the autoradiograph.

One of the primary objectives of this work was to identify proteins that are vital either in killer cell activation or the lytic mechanism itself. An essential prerequisite for such involvement is the demonstration that the mRNAs are induced upon CTL activation prior to measurable cytotoxic activity. A series of cytodots were prepared from spleen cells activated with either irradiated stimulator cells or Con A. The level of cytotoxicity, measured in a chromium-release assay, peaked 24 hr after the maximum expression of B10 and C11. In addition, the levels of B10 and C11 declined rapidly as cytotoxicity began to decline. However, the cultures still showed high levels of cytotoxicity, even after the mRNAs encoded by these two clones had returned to background levels. Once the genes have been activated and the transcripts have been translated, there is no reason why the mRNAs should be stable in the cells, as the phenotype (i.e., cytotoxicity) maintained is a reflection solely of the proteins present. Most importantly, the primary prerequisite that gene activation precedes phenotypic expression is satisfied for both B10 and C11. Final proof of the importance of these genes can only come through the discovery of the biological activities of the proteins they encode and comparison of these proteins with those that comprise the cytotoxic granules described by others (Henkart *et al.*, 1984; Podack and Konigsberg, 1984).

In summary, we have isolated two gene sequences that are expressed exclusively in cytotoxic T lymphocytes. The clones represent very useful probes with which to study the events that control gene expression during T cell ontogeny from the pluripotent precursor to the ultimate end point of activation.. In addition, the relationship between the two sequences and the mRNAs detected poses some interesting genetic questions. A knowledge of the protein sequences that they encode should provide a more precise

definition of T cell subsets. In combination with other T cell markers, these should allow further dissection of the complex cellular networks of the immune system and enable the identification of active cytotoxic T lymphocytes in tissue sections and in normal and leukemic populations of cells. The pattern of expression is certainly in keeping with the hypothesis that these sequences encode proteins that are important either in T cell activation or T cell cytotoxicity. The primary responses studied showed a transient burst of specific mRNA transcription followed by a longer wave of protein expression as indicated by the level of cytotoxicity. In contrast, in two T cell lines that constantly exhibit cytotoxic activity, the mRNAs detected by the two probes appeared to be transcribed constitutively. Knowledge of the molecular basis of CTL functioning should be of great interest to immunologists and may initiate development of innovative forms of immunotherapy.

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CHAPTER III

NOVEL SERINE PROTEASES ENCODED BY TWO CYTOTOXIC T LYMPHOCYTE-SPECIFIC GENES[1]

A. INTRODUCTION

Cytotoxic T lymphocytes (CTL's), also referred to as T killer cells, are effector cells in cell-mediated immune reactions. They specifically recognize foreign antigens on the surface of target cells, bind to them, and cause the target cells to lyse. Although the various steps in this process have been analyzed in considerable detail (Berke, 1983; Nabholz and MacDonald, 1983), most studies have not provided insight into the mechanism by which the killer cell effects the lysis of a target cell. A means of identifying relevant molecules, based on cloning CTL-specific genes, has been described (chapter II; Lobe *et al.*, 1986). The transcripts corresponding to two of these genes (B10 and C11) were detected exclusively in activated CTL's.[2] Moreover, the kinetics of messenger RNA (mRNA) expression, as detected by these two cloned probes, closely paralleled but preceded cytotoxicity throughout cytotoxic responses *in vitro* (Lobe *et al.*, 1986).

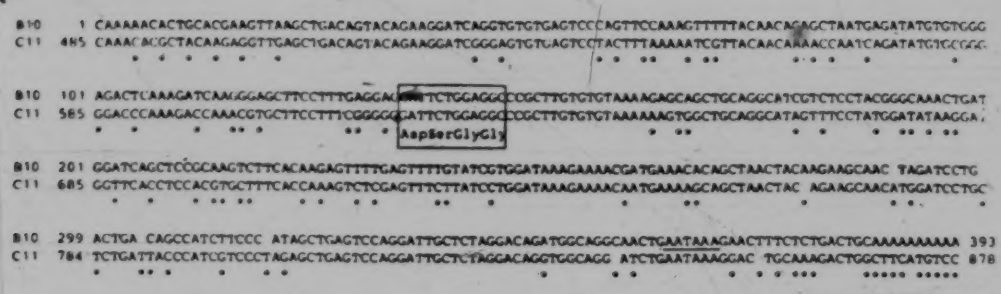
B. RESULTS AND DISCUSSION

Sequence analysis (Sanger *et al.*, 1980) of B10 and C11 (figure 1A) revealed that they were related to each other and that the hypothetical proteins they encode contain a short region characteristic of serine proteases, Asp-Ser-Gly-Gly (a sequence homologous to that surrounding Ser^{195} of chymotrypsin). With B10 and C11 as probes, another CTL

[1] A version of this chapter has been published. Lobe, C.G., Finlay, B.B., Paranchych, W., Paetkau, V.H., and Bleackley, R.C. (1986). *Science* **232**:858-861.

[2] To date, B10 and C11 expression has been detected in seven of seven cloned CTL lines and in none of three cloned T-helper cell lines. Other cell types tested, including murine brain, liver, thymus and lipopolysaccharide-activated B cells and human CTL's, gave no signal (C.Lobe, unpublished data).

A



B

FIGURE III.1 (A) Nucleotide sequence comparison of clones B10 and C11. Insert DNA was purified from the CTL-specific clones designated B10 and C11, recloned in M13 or pUC vectors and sequenced by the dideoxy method (Sanger *et al.*, 1975). The two sequences were maximally aligned; * designates nucleotide mismatches. The sequence of B10 is numbered from the first nucleotide of the insert, whereas that of C11 has been renumbered to correspond with the full-length sequence shown in (B). The region encoding the characteristic serine protease sequence Asp-Ser-Gly-Gly is boxed, and the polyadenylaton signal sequence of B10 is underlined. (B) Nucleotide and predicted protein sequence of C11 insert. A size selected cDNA library (>1000 base pairs) was screened with a C11 insert isolated from the original library (chapter II; Lobe *et al.*, 1986). A plaque that contained a cross-hybridizing sequence, whose apparent length was 1400 base pairs was selected for further analysis. Restriction fragments were subcloned in M13 or pUC13 and sequenced as above. A potential initiator methionine codon is present at nucleotide 16 followed by an open reading frame to nucleotide 756. A putative ribosome binding site (- - - - -) is underlined. By analogy with RMCPII (see text and figure 2), the numbering of the C11 protein starts at the isoleucine 21 residues downstream from the proposed translational initiation site. The amino acids which form the catalytic triad of the serine proteases (His^{57}, Asp^{102}, and Ser^{195} in chymotrypsin) are marked ( ).

complementary DNA (cDNA) library was screened, in which inserts greater than 1000 base pairs were cloned in lambda gt10. Forty thousand recombinants were screened and 39 plaques corresponding to C11 were isolated, but no evidence for a B10 recombinant could be found.

A cDNA insert of 1400 base pairs, which hybridized with C11, was selected for sequence analysis (Sanger *et al.*, 1980). The predicted protein sequence encoded, of molecular weight 25,319 is shown in figure 1B. The putative start codon is preceded by a potential ribosome binding site CCUUCCG (Hagenbuchle *et al.*, 1978), and a polyadenylation signal sequence AAUAAA (Proudfoot and Brownlee, 1976) occurs just upstream from the poly(A) tract. Of the first 12 amino acids predicted, ten are hydrophobic, and the amino acid in position 2 (Lys) is basic, suggesting that this sequence may act as a signal to direct secretion or intracellular organelle location (von Heijne, 1985).

A search of the National Biomedical Research Foundation (NBRF) protein sequence data bank revealed that the protein encoded by C11 resembles a number of serine proteases (Table 1). When the sequences were optimally aligned according to the Dayhoff algorithm (Dayhoff, 1979), the homologies generally varied between 30 and 40 percent. The greatest homology was found with rat mast cell protease type II (RMCPII), which had amino acids identical to 109 of 215 amino acids encoded by C11, giving a match per length of 51 percent. The amino acid residues known to form the catalytic triad of the active site in serine proteases (His^{57}, Asp^{102}, and Ser^{195}) (Neurath, 1984) were all found in the protein encoded by C11. The sequences around these residues, which are highly conserved among serine proteases, are also conserved in the C11 gene product. Indeed, largely because of conservation around this region, the protein encoded by C11 appears to be somewhat homologous (about 30 percent of 209 residues) even to the prokaryotic proteases trypsin and type B from *Streptomyces griseus*.

The cytotoxic T cell specific proteins (CCP's) encoded by C11 and B10 will be referred to as CCPI and CCPII, respectively. In figure 2 the optimal protein alignment

Protein (EC #)	Organism	Residues Compared C11-Protein (CCPI)	Bank-protein	% Homology
7S nerve growth factor (EC 3.4.21)	Murine	29-224	26-229	40
Chymotrypsin A (EC 3.4.21.1)	Bovine	1-200	16-216	35
Chymotrypsin B (EC 3.4.21.1)	Bovine	1-200	16-216	36
Complement C1r (EC 3.4.21.41)	Human	52-224	56-238	35
Elastase (EC 3.4.21.11)	Porcine	3-220	3-233	33
Factor X (EC 3.4.21.6)	Bovine	1-225	192-421	33
RMCP II (EC 3.4.21)	Rat	1-214	1-213	51
Kallikrein (EC 3.4.21.8)	Rat	26-225	51-262	36
Plasminogen (EC 3.4.21.7)	Human	3-224	563-787	37
Plasminogen Activator (EC 3.4.21.31)	Human	72-224	389-560	35
Trypsin (EC 3.4.21.4)	Streptomyces griseus	29-220	22-214	33
Trypsin (EC 3.4.21.4)	Rat	29-226	31-228	39

TABLE III.1 A selection of proteins that are homologous to the predicted C11 protein, CCPI. The protein sequence predicted from the longest open reading frame encoded by CCPI was compared with the National Biomedical Research Foundation protein sequence data bank. The numbering for CCPI (see text) is given in figure 1B. The data bank numbering system has been used for homologous proteins. All of the proteins that were significantly homologous (>30%) with CCPI over a large portion of the molecule (>150 residues) were serine proteases.

with CCPI is presented for RMCPII, bovine chymotrypsin, bovine trypsin, and CCPII. RMCPII is an intracellular serine protease found in the granules of atypical mast cells (Woodbury *et al.*, 1978a; Woodbury and Neuurath, 1980). The high level of homology of CCPI with RMCPII is particularly intriguing as RMCPII has a number of structural features that make it exceptional in the serine protease superfamily (Woodbury *et al.*, 1978b). Protein CCPI contains cysteines in precisely the same positions as RMCPII which, by analogy with RMCPII, form three disulfide bonds. These occur in the same positions in chymotrypsin, trypsin and elastase. Both CCPI and RMCPII lack a disulfide bond that is present in all other known serine proteases, including several from prokaryotes (Johnson and Smillie, 1974; Jurasek *et al.*, 1974), and that links Cys^{191} with Cys^{220} in chymotrypsin. In both CCPI and RMCPII the first of these two half-cystines is replaced by a phenylalanine, while the second half-cystine has been deleted along with other residues. Linkage of $Cys^{191}$to Cys^{220} is thought to be important in stabilizing the conformation of the substrate binding site (Woodbury *et al.* , 1986b). Its absence in CCPI and RMCPII may lead to significant changes in that site and, hence, in substrate specificity.

Two other primary structure changes previously seen only in RMCPII and thought to alter substrate binding are also present in the predicted CCPI protein. In RMCPII and CCPI the amino acid six residues before the active-site serine is alanine. In chymotrypsin-like proteases it is serine and in trypsin-like proteases, aspartic acid. The residue in this position lies at the bottom of the S_1 binding site[3], so the change to a less polar residue would indicate a preference for a hydrophobic amino acid at the P_1 position in the substrate (Johnson and Smillie, 1974; Jurasek *et al.*, 1974). Furthermore, the sequence Ser-Trp-Gly^{216} in chymotrypsin, which forms hydrogen bonds with the P_1 and P_3 residues of the

[3] The peptide bond cleaved by proteases is between residues P_1 and P_1'. The corresponding amino acids in the protease are designated S_1 and S_1'. Other residues are numbered relative to the peptide bond cleaved (Segal *et al.*, 1971).

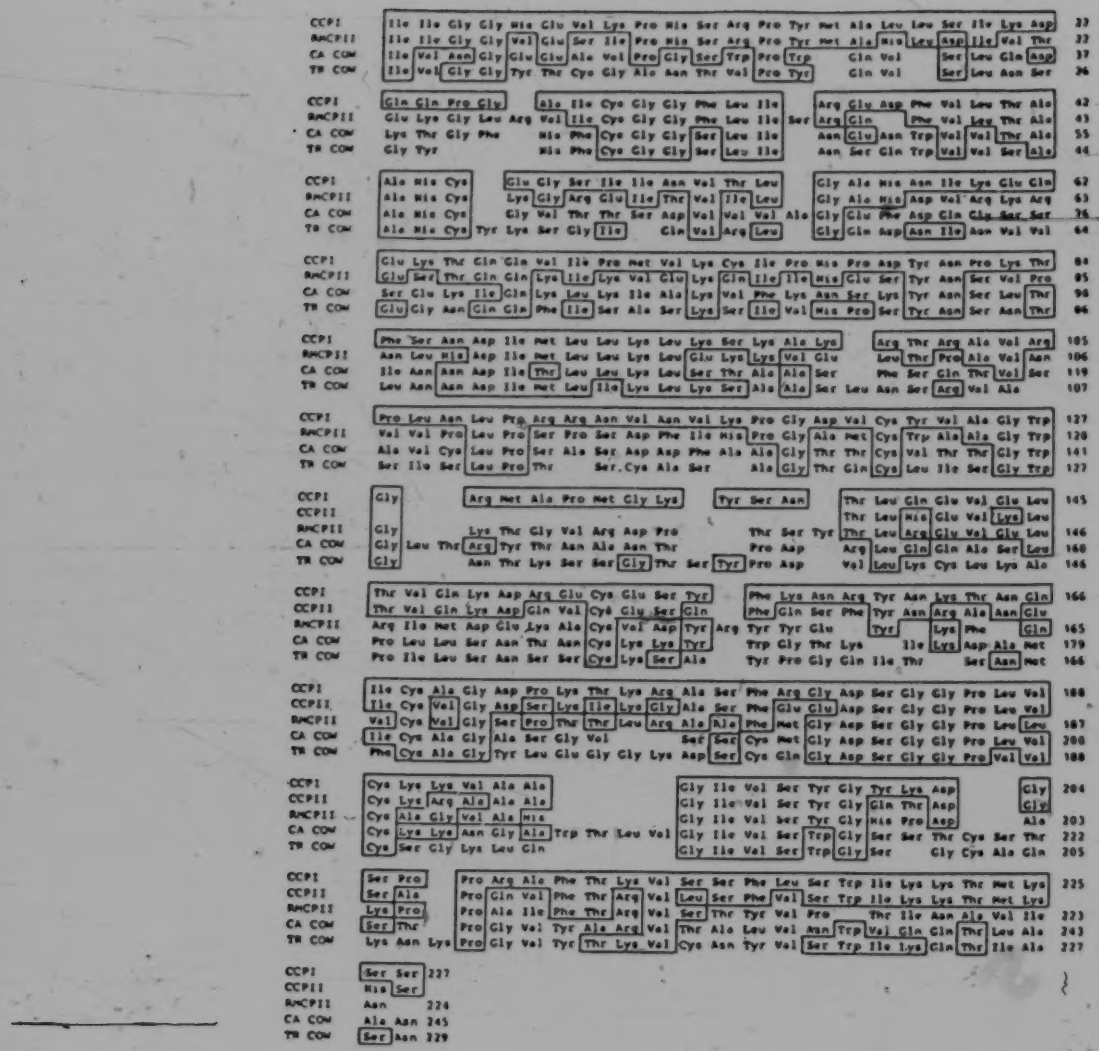


FIGURE III.2 Alignment of the predicted CCPI protein sequence with other serine proteases. The protein sequence predicted from C11 is aligned, according to the method of Dayhoff (1979), with rat mast cell protease (RMCPII), bovine chymotrypsin (CA COW), bovine trypsin (TR COW), and the partial protein sequence predicted from insert B10 (CCPII). Amino acids that are identical to those of CCPI are boxed. Residue numbers are given at the end of each line; CCPI and RMCPII start at the Ile residue, whereas the numbering of CA and TR is based on the inactive zymogens. No numbering is presented for CCPII because the full sequence has yet to be determined.

substrate[3], is replaced by Ser-Tyr-Gly in CCPI and RMCPII, again suggesting altered substrate secificity. Both of these changes are also seen with CCPII.

One of the few RMCPII-specific differences that is not present in CCPI is the substitution of isoleucine at position 99 in chymotrypsin for asparagine. In most mammalian serine proteases this residue is hydrophobic, and indeed in CCPI it appears to be phenylalanine. However, most of the RMCPII-specific changes are present in CCPI protein, suggesting that the substrate binding site of CCPI will resemble that of RMCPII and could be significantly different from those of other mammalian serine proteases (Powers *et al.*, 1985).

III. CONCLUSIONS

In addition to the interesting aspects of the structure of CCPI, the fact that both CCPI and CCPII appear to be serine proteases has significant implication for the mechanism by which CTL's are activated to become capable of lysing target cells (Berke, 1983; Lobe *et al.*, 1986). Protease inhibitors have been shown to block T cell effector function (Redelman and Hudig, 1980; Chang and Eisen, 1980), and a trypsin-like esterase activity was shown to be induced during CTL activation (Pasternack and Eisen, 1985). We have demonstrated that two genes that encode protease-like proteins are expressed in CTL's. Previous evidence that these genes are activated specifically in CTL's and that their expression correlates with cytolytic activity (Lobe *et al.*, 1986) suggests that they play a key role in the development of a cytotoxic response.

The two genes we have so far characterized appear to encode serine esterases, and another CTL-specific gene, AR10, cloned by Gershenfeld and Weissman (1986) encodes a trypsin-like enzyme. These findings suggest that a protease cascade mechanism may be involved in T cell cytotoxicity. Such a mechanism has been shown in a number of biological systems, including blood coagulation and complement-mediated lysis, and is

advantageous in control, specificity, and amplification of enzymatic reactions (Neurath and Walsh, 1976).

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APPENDIX TO CHAPTERS II & III

A. INTRODUCTION

In chapter II, the isolation of two CTL-specific cDNA clones was described and the correlation of their expression with cytotoxic activity was established. The DNA sequences and putative protein products, cytotoxic cell protease (CCP) I and II, were presented in chapter III. The possible role of the serine protease products in cytolysis by CTL was also discussed. Further experiments have been carried out which support the initial findings and give further clues as to the function of these molecules. The methods used are as described in chapters II and III. The CTL-specific expression of B10 and C11 was confirmed by extending the analysis to several additional cell lines, including NK, helper T cell lines, macrophages, S194 myeloma cells and fibroblasts. The correlation of transcription of B10 and C11 genes with the lytic activity of CTL was also verified by monitoring the level of expression of B10 and C11 in long-term MLR cultures, in an antigen-dependent CTL line and antigen-dependent cytotoxic/helper T cell lines, all of which have inducible killing activity. The intracellular localization of the C11 protein was investigated using antibodies against the predicted sequence. Finally, two new genes were identified which appear to be additional members of the family which includes B10 and C11. The specificity of expression of the transcripts corresponding to these sequences was determined.

B. RESULTS & DISCUSSION

1. *B10 and C11 are CTL-specific*

The restriction of B10 and C11 expression to cytolytic T cells was verified and extended to include a wider panel of cell types (figures 1 and 2). In addition to the data presented in chapter II for B10 and C11, figure 1 shows the results for NK and memory CTL.

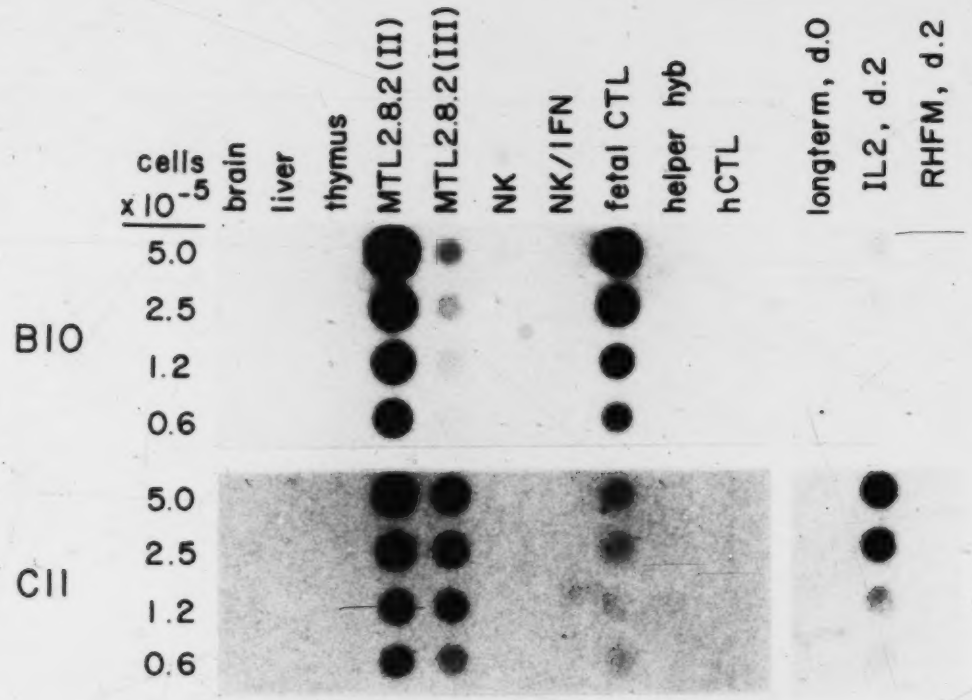

FIGURE IIIA.1 Tissue specificity of B10 and C11 expression. A cytoplasmic dot blot of total RNA was hybridized with nick-translated B10 (upper panel), washed, and then hybridized with C11 (lower panel). The cells tested are indicated at the top: MTL2.8.2 (II) and (III) are mouse CTL cell lines; NK are Percoll-gradient purified NK cells; NK/IFN are the same cells stimulated with 1000 U/ml IFN; fetal CTL cells were derived from murine fetal thymus; helper hyb. is an antigen-specific helper hybridoma, and hCTL are human peripheral blood lymphocytes, stimulated with alloantigen. The final 3 lanes represent cells from a memory CTL reaction, resting (d.0), and 2 days after stimulation with IL2 (IL2 d.2) or with media alone (RHFM d.2). The number of cells is shown at the side.

A population of natural killer (NK) cells were prepared by Percoll gradient centrifugation of spleen cells (Timonen and Sakesela, 1980; carried out by Bill Pohajdak). These cells had low cytotoxic activity against the NK tumour cell target YAC-1, at an effector:target ratio of 20:1. RNA was isolated from them and cytodots were prepared and hybridized with radioactive B10 or C11 probe (figure 1). Neither B10 nor C11 were expressed at a detectable level in these cells. Stimulation of the NK cell fraction with 1000 U/ml of gamma-interferon for 17 hrs increased their cytotoxic activity 5.5-fold (from 6% to 33% lysis of the YAC-1 cell target at an effector:target ratio of 20:1). However, even these stimulated NK cells did not appear to express B10 or C11. When the cytodot was hybridized with an actin gene probe, all of the RNA samples on the cytodot gave a radioactive signal (data not shown). Therefore we conclude that NK cells do not synthesize C11 or B10 transcripts, even when they have high levels of cytotoxic activity. This implies that the C11 and B10 products are not required for NK cytolytic function and, possibly, some other serine proteases are utilized instead. Interestingly, several NK cell lines which have been tested (NK-3, NK-1AK and 1B10) are positive for C11 (J. Kaptein and G. Dennert, personal communication). It is possible that these "NK lines" are CTL-lines which have lost their antigen specificity.

The level of B10 and C11 expression in memory CTL restimulated with IL2 was tested in a long-term MLR culture. The secondary CTL present in these MLR cultures, after two rounds of antigenic stimulation, have returned to a quiescent state, but cytotoxic activity can be regenerated by stimulation with IL2. This response is apparently antigen-independent and can occur in the absence of cell proliferation (Bleackley *et al.*, 1985; Lefrancois *et al.* 1984). To monitor B10 and C11 expression in this type of response, spleen cells from CBA/J mice were initially stimulated with irradiated F(1) target spleen cells, then allowed to return to a resting, non-cytolytic state for 18 days. At this point a cell sample was taken for RNA preparation. The cells were then re-stimulated by addition of fresh media containing IL2, which increased the cytolytic activity of the cultures 10-fold

after 2 days. A change of media alone, without IL2, stimulated a 2- to 3-fold increase in cytotoxic activity (Jonathan Hooten, personal communication). RNA was prepared from the cells 2 days after incubation in fresh media with or without IL2. A cytodot of the RNA samples was prepared and probed with the cDNA clones. The B10 and C11 transcripts were not detectable after the 18 day period (figure 1, "long term d.0"), but are apparent after IL2 stimulation (figure 1, "IL2 d.2"). The control of a change of media without IL2 gives no stimulation of B10 or C11 expression (figure 1, "RHFM d.2").

Two more murine helper cell lines were tested, a helper hybridoma (Teh *et al*, 1985; figure 1) and CH2.4 (C. Havele, unpublished; figure 2), both of which were negative for C11 and B10. A lymphoma, RI, and a B cell myeloma, S194, were also negative for both clones (figure 2). Macrophages, which have the ability to lyse certain target cells, also had no detectable level of B10 or C11 transcript (data not shown). When probed with the actin gene, all of these RNA samples were positive and gave a comparable level of hybridization to the MTL2.8.2 positive control. Another non-lymphoid cell type tested was an L929 fibroblast line, which was also negative for C11 and B10 expression (figure 3A).

The CTL line MTL21.9(I) is an $H2^k$ anti-$H2^b$ clone which is antigen- and IL2-dependent (Havele *et al.*, 1986).[1] For optimal growth, these cells require antigen-stimulation approximately every 2 weeks, whereupon they proliferate and increase in size and in cytolytic activity. The level of B10 and C11 mRNA in these cells was analyzed by Northern blotting (figure 2). Resting MTL21.9 had a low level of the C11 transcript relative to antigen-independent CTL-lines. The level increased 5-fold after the cells were stimulated with antigen. B10 was also expressed, but at a very low level, in the resting type I cells. With a longer exposure of this Northern blot, a signal for B10 was seen for resting MTL21.9(I) (data not shown). As with C11, the level of the B10 transcript increased after antigen stimulation. Thus, as was previously seen for alloantigen-stimulated

[1]The "I" designation following the name of the cell line indicates it is antigen- and IL2-dependent. Type "II" cells have lost their antigen requirement, and type III cells are both antigen- and IL2-independent.

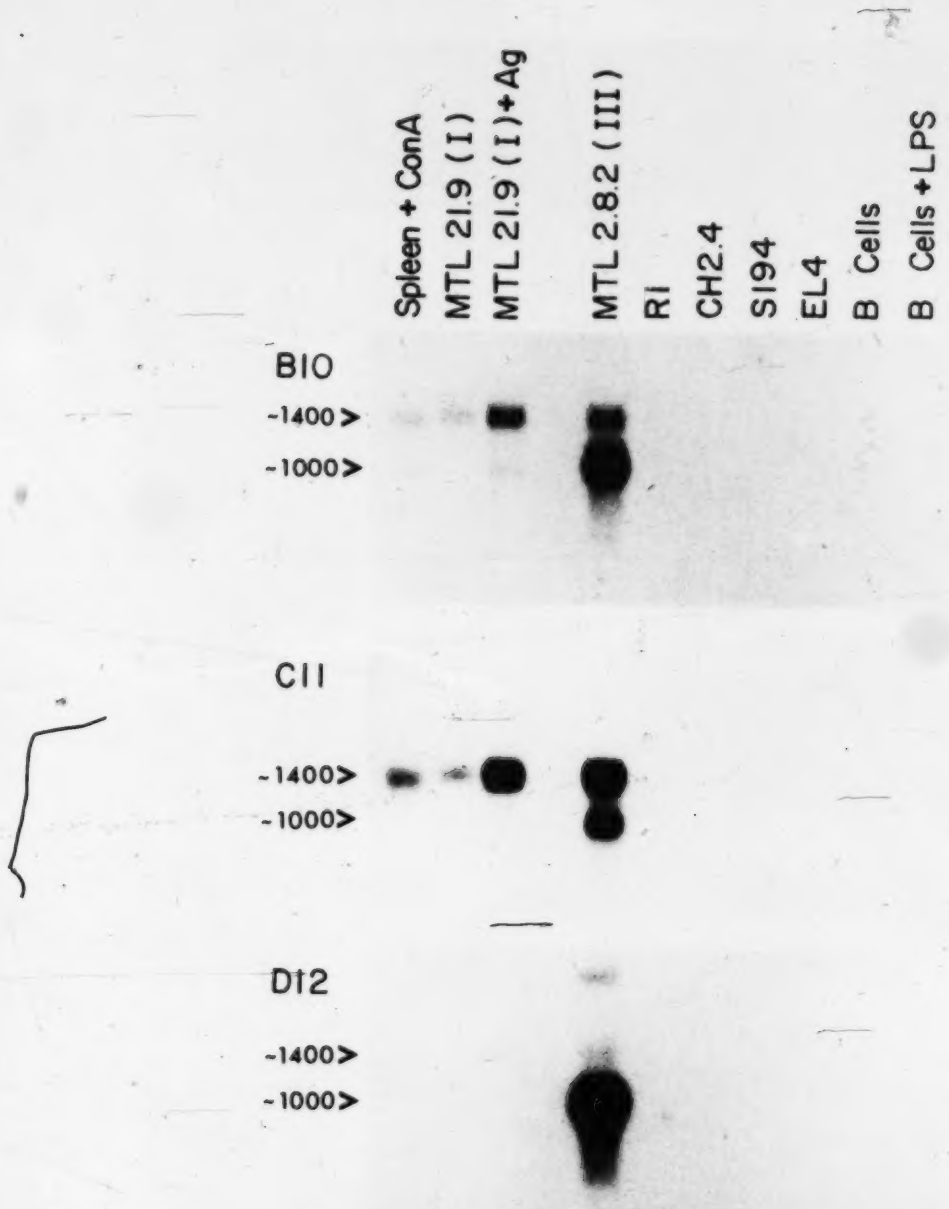

FIGURE IIIA.2 Northern blot analysis of B10, C11 and D12 expression. Total RNA was prepared from cells: spleen+ConA, mouse spleen cells 3 days after ConA stimulation; MTL21.9(I), an antigen-dependent CTL line, resting; MTL21.9(I)+Ag, the same cell line 3 days following antigen stimulation; MTL 2.8.2(III), a CTL line; RI, a T cell lymphoma; CH2.4, an antigen-specific helper T cell clone; S194, a B cell myeloma; EL4, a lymphokine-producing T cell thymoma. For the last two lanes spleen cells were enriched for B lymphocytes by nonadherance to Petri dishes and anti-Thy1.2 treatment, and RNA was prepared from resting B cells or from LPS-stimulated B cells. In all cases RNA was size-fractionated on a 0.8% formaldehyde agarose gel, transferred to nitrocellulose and probed sequentially with nick-translated B10, C11, and finally D12. In the B10 hybridization (upper panel), the 1000 ntd B10 transcript is indicated; the higher MW band is due to cross-hybridization to C11. Similarly, in the C11 hybridization, the 1400 ntd C11 transcript is indicated and cross-reactivity to the B10 transcript is seen for MTL2.8.2(III) cells.

spleen cells (chapter II; Lobe *et al.*, 1986a) and splenic MLR cultures, the level of expression of B10 and C11 in a type I CTL line correlates with cytolytic activity. The relative level of expression is also similar to activated spleen cells in that the B10 transcript is at a much lower abundance than C11, whereas in type II (antigen-independent) CTL lines, the abundance of the B10 transcript is as high as C11. In the upper panel of figure 2, the band corresponding to the B10 transcript is indicated with an arrowhead; the higher MW band is due to cross-hybridization with C11. Similarly, C11 cross-hybridization to the B10 transcript is seen in the middle panel, MTL2.8.2(III) lane[2].

2. *B10 and C11 transcripts are sequentially induced*

The induction of B10 and C11 expression was analyzed using a Northern blot of the RNA samples, rather than a cytodot, so that a more accurate determination of the time course of their expression could be obtained. On a cytodot, the specific signal cannot be discerned from background signal which is due to cross-hybridization of homologous sequences. On a Northern blot the RNA is size-separated, so the signals due to homologous but different-size transcripts, such as B10 and C11, can be distinguished. Spleen cells were stimulated with concanavalin A (ConA) and RNA was isolated at 1 to 4 days post-stimulation, as described in section II.C.4 except that the RNA was run on a Northern, rather than a cytodot. The Northern blot was then hybridized with B10 and C11 (figure 3). The band corresponding to C11 began to appear at day 2, peaked at day 3, and was almost undetectable at day 4. The B10 mRNA, however, did not appear until day 3 and peaked at day 3 to 4, ~12 hrs later than C11. Therefore, C11 and B10 appear to be sequentially regulated.

[2]Conditions in which B10 and C11 do not cross-hybridize were established subsequent to these experiments. It requires a very stringent hybridization in a 50% formamide solution at 50 C, followed by washing in 0.1XSET-0.1%SDS at 65 C.

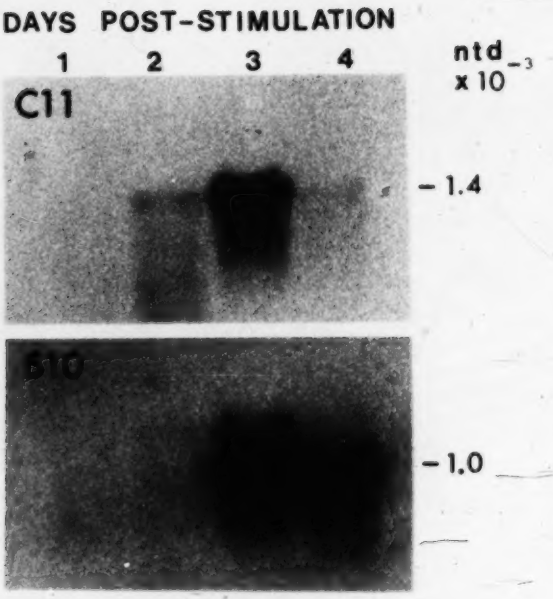

FIGURE IIIA.3 Time-course of C11 and B10 expression. Total RNA was isolated from spleen cells at days 1 to 4 after being stimulated with ConA. A Northern blot was prepared as described in figure IIIA.2, hybridized with nick-translated C11, then washed and hybridized with nick-translated B10. The exposure for the C11 hybridization was 1 day, and for B10, 6 days. (Northern blot prepared by J. Shaw)

3. B10 and C11 are expressed in helper T cell clones with cytolytic activity

Target cell lysis, traditionally ascribed only to killer T cells (T_K), has now also been demonstrated in some T_H cell lines (Tite *et al.*, 1985; Tite and Janeway, 1984). Two helper cell clones, 21C11 and 153E6 (Lyt1$^+$, L3T4$^+$), were tested for B10 and C11 expression. These cells were of interest because although they have the surface phenotype of T_H cells and produce lymphokines (IL2 and gamma-interferon), they also have CTL-like granules, produce lymphotoxin (LT) and have cytolytic activity (Schmid *et al.*, 1986). Both lines are antigen- or mitogen-dependent for growth, but their cytolytic activity peaks much faster (10 to 12 hrs after ConA-stimulation) than the MTL21.9(I) T_K clone (2 to 3 days post-antigen). Slot blots and a Northern blot of RNA from these cells were prepared by Dr. N. Ruddle (Yale). One set of samples represents RNA from cells which had been stimulated with ConA for 12 hours (figure 4A slot blot and figure 5 Northern). The other set is of RNA from a time-course experiment in which cells were incubated with ConA or ConA+cyclohexamide and samples taken at 0, 3 and 7 hours (figure 4B slot blot). These blots had previously been probed with a lymphotoxin genomic sequence. When probed with C11, RNA from 153E6 and 21C11 T cells 12 hrs after ConA stimulation was positive for the C11 transcript, both on the RNA slot blots (figure 4A) and Northern blot (figure 5). The blots were then washed and probed with B10, which gave no detectable signal on the slot blot, but did reveal a low level of the B10 transcript in the 21C11 RNA on the Northern blot (figure 7). This low level of B10 expression relative to C11 is similar to the situation in stimulated spleen and antigen- and IL2-dependent CTL. There is also some cross-hybridization to the larger C11 transcript. When the filters were subsequently probed with another related cDNA clone, D12 (described in section B.8), a low level of hybridization was seen for the 21C11 RNA, both on the slot blot and the Northern blot. The D12 probe is also weakly cross-hybridizing to the C11 transcript. The negative control cell line, L929 fibroblasts, was negative for all three probes. When these blots were

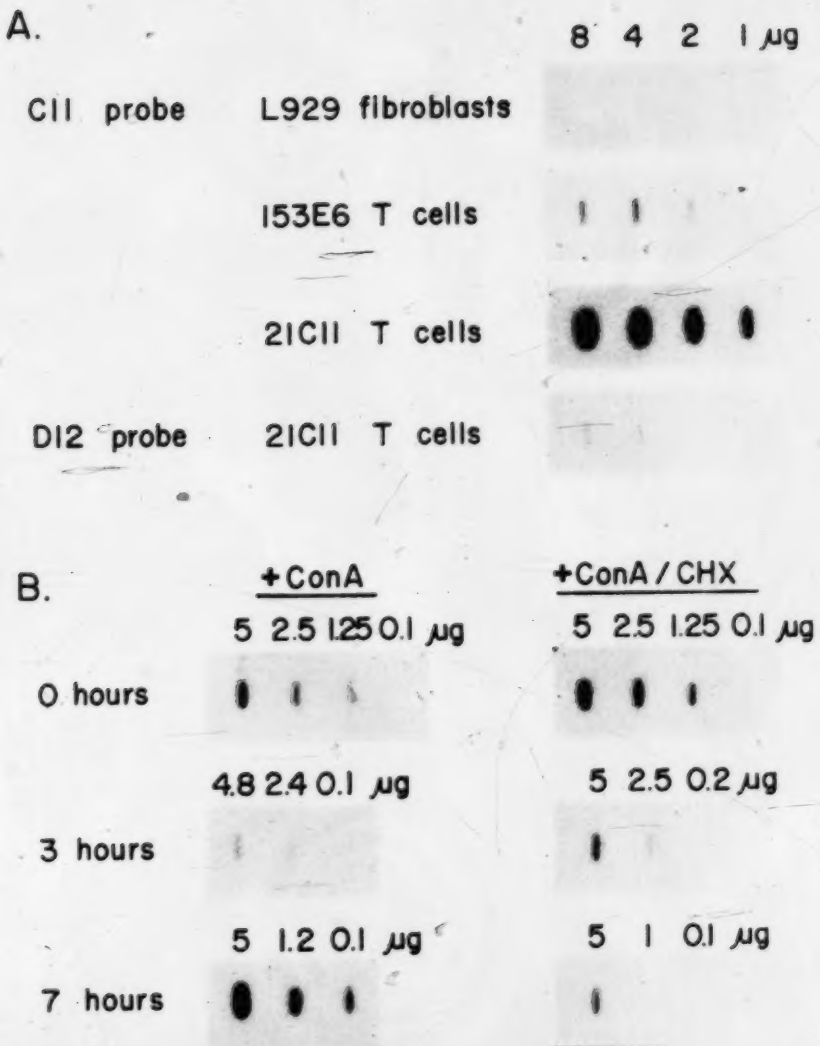

FIGURE IIIA.4 Expression of C11 and D12 in cytotoxic T helper cells. A. Slot blots of RNA prepared from a fibroblast line, L929, and two helper/killer T cell clones, 21C11 and 153E6, each after 12 hrs incubation with ConA. The blots were hybridized with C11 and D12 cDNA insert. Only the 21C11 T cell results are shown for D12. B. The 21C11 cells were incubated at 2 x 10^6 cells/ml with 5 ug/ml ConA and with or without cycloheximide addition (5ug/ml) and samples taken at 0, 3 and 7 hr, as indicated at the side. The results are shown for the hybridization with nick-translated C11 insert DNA. The amount of RNA in each slot is shown above it. (RNA blots provided by N. Ruddle)

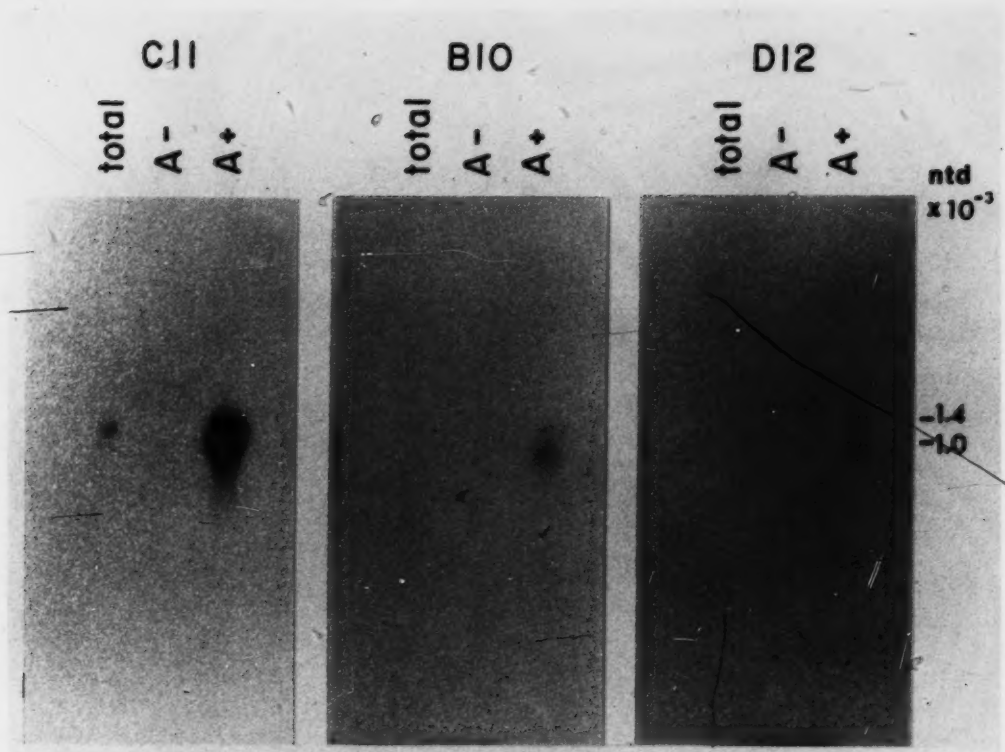

FIGURE IIIA.5 Northern blot of RNA from the $T_{H/K}$ cell clone, 21C11, (provided by N. Ruddle). The blot was probed in turn with nick-translated C11, B10 and D12 cDNA insert. The lanes contain 10 ug each of total cytoplasmic RNA, poly A^- RNA, and poly A^+ RNA.

probed with a lymphotoxin DNA sequence, the results were the same as for C11 - the 21C11 and 153E6 $T_{H/K}$ lines were positive and the fibroblast line was negative.

The kinetics of induction of the increase in the level of the C11 transcript in the ConA-stimulated 21C11 cells is shown in figure 4B. The level of C11 mRNA was quite high by 7 hours after incubation with ConA, which was also seen when the lymphotoxin gene was used to probe this blot. The characteristic cytolytic granules are easily visualized in the cells and contain perforin by that time (Dr. N. Ruddle, personal commun.). The similar kinetics of induction of C11 and LT suggests that their expression is activated by a common signal. However, when cyclohexamide (which inhibits mRNA translation) was included in the ConA incubation, this rise in the C11 mRNA appeared to be blocked, whereas for lymphotoxin mRNA the induction was enhanced (Dr. N. Ruddle, personal commun.). The lack of an increase in C11 mRNA when *de novo* protein synthesis was blocked implies that an activator protein must be synthesized for C11 regulation. For LT mRNA, the enhanced induction in the absence of protein synthesis suggests that at least part of LT regulation occurs via synthesis of an inhibitor of LT mRNA accumulation. This most likely corresponds to an RNase activity which, by analogy to the regulation of lymphokine expression, rapidly degrades the mRNA (see chapter I, section B.1.d; Shaw and Kamen, 1986). The fact that LT mRNA levels are superinduced in the presence of cyclohexamide means that LT gene transcription must still be occurring. Thus the signal responsible for activation of the LT gene must be resistant to cyclohexamide and therefore different from the one for C11.

4. *Correlation of B10 and C11 expression with esterase and cytolytic activity*

The level of B10 and C11 transcription in four different $T_{H/K}$ cell clones was assayed. The T cell clones were originally generated as antigen-specific helper T cell lines and some were subsequently found to possess cytolytic activity (Tite *et al.*, 1985). The presence of serine esterase activity in these cells was determined by a benzyoxycarbonyl

lysyl thioester (BLT) assay, which detects serine esterases with trypsin-like specificity (Pasternack and Eisen, 1985).

Each of the cell lines was stimulated with ConA and a sample was taken for RNA isolation at 0, 2, 4, 8 and 16 hrs post-stimulation. The level of C11 and B10 transcripts was then measured by cytodot analysis (figure 6). C11 was expressed in all of the clones, with a peak at 4 to 16 hrs, depending on the cell line. B10 transcription was induced in the 5.5 and 5.9 T cell clones (confirmed on a Northern blot, data not shown). It may also be expressed in the 5.8 T cell clone, but the amount of 5.8 T cell RNA analyzed on a Northern blot was too low to determine if the cytodot signal was due to a B10 transcript or to cross-hybridization to the C11 transcript. B10 was not expressed in the D10 T cell clone -- the cytodot signal was due to cross-hybridization to a higher MW transcript, probably C11, as determined on a Northern blot (data not shown).

The results are summarized in Table II. For each of the clones which possesses cytotoxic activity, both B10 and C11 seem to be expressed. The D10 T cell line, which is not cytotoxic, expresses C11 but not B10. The lack of cytotoxic activity in these cells may be due to the absence of B10. It is also possible that the C11 mRNA is not being translated to a functional protein. There is no correlation of B10 or C11 expression to the serine esterase activity. However, the BLT-assay detects trypsin-like esterase activity, which probably corresponds to the CTL-specific AR10-encoded protein (Gershenfeld and Weissman, 1986). The B10- and C11-encoded proteins were predicted to have a very unusual substrate specificity (chapter III; Lobe *et al.* 1986b) which would not be detectable in this assay. Therefore C11 and B10 expression correlated with cytotoxic activity; BLT-esterase activity did not correlate with B10 or C11 expression, nor, in fact, to cytolytic activity.

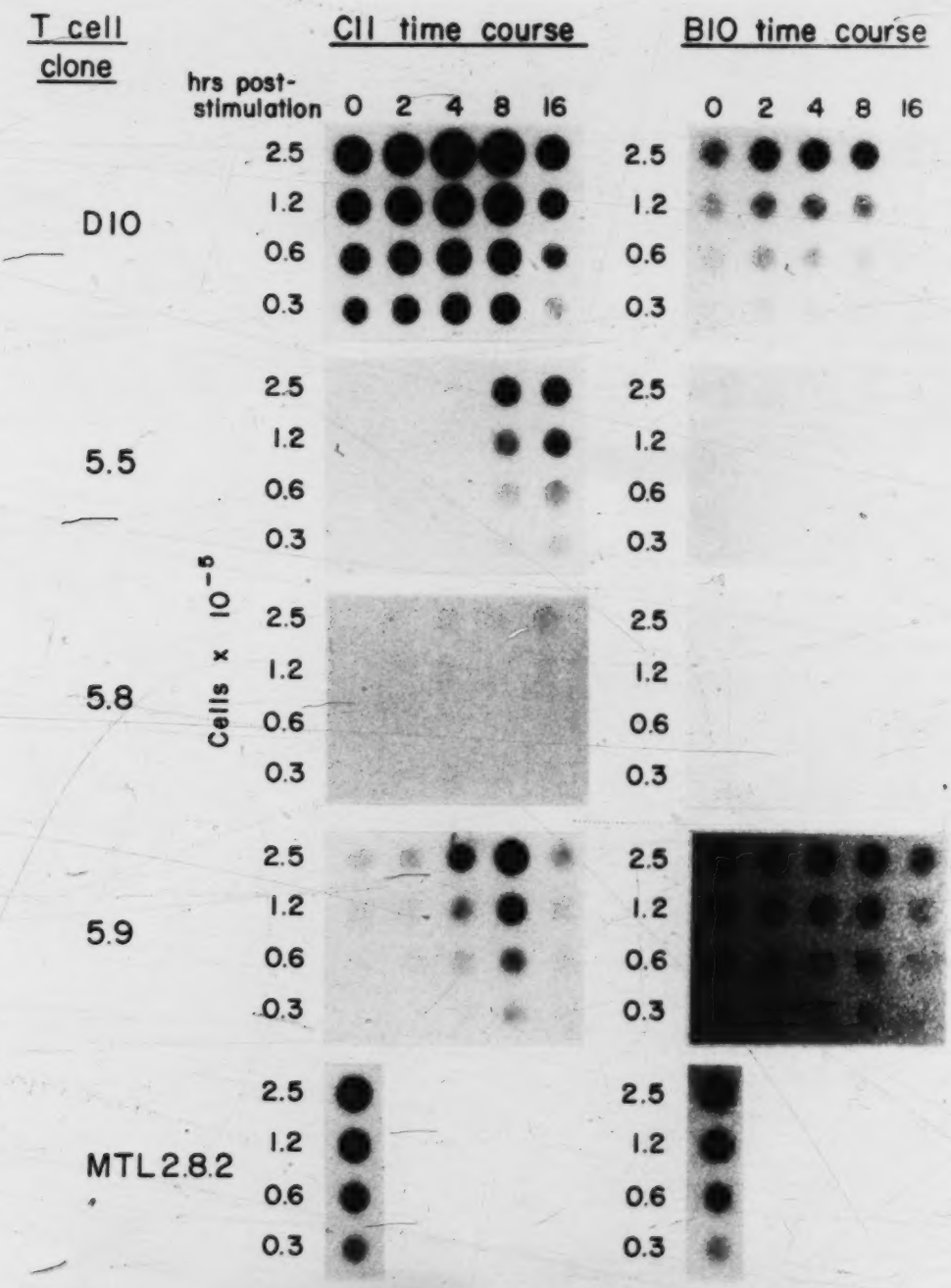

FIGURE IIIA.6 Expression of B10 and C11 in T cell clones with or without esterase activity. The helper T cell clones, D10, 5.5, 5.8 and 5.9, were stimulated with ConA and samples were taken after 0, 2, 4, 8 and 16 hrs for RNA preparation. RNA cytodots were prepared and hybridized with C11 and B10 cDNA insert. RNA from MTL2.8.2 cells was included on the cytodot as a positive control (bottom panels). The time after stimulation is indicated along the top, and the number of cell equivalents X 10^{-5} along the side. The comparison of these results with cytolytic and esterase activity of the T cell clones is shown in Table IIIA.1.

T cell clone	D10	5.5	5.8	5.9	MTL2.8.2
cytolytic activity	-	+	+	+	low
serine esterase activity (BLT)	+	+	-	-	+
C11 expression	+	+	+	+	+
B10 expression	-	+	?	+	+

TABLE IIIA.1 Comparison of cytolytic activity, esterase activity and C11 and B10 expression. The T helper cell clones, D10, 5.5, 5.8 and 5.9 were each assayed for cytolytic activity in a ^{51}chromium release assay and serine esterase activity by a BLT chromatographic assay (Pasternack *et al.*, 1986). The same cells were assayed for C11 and B10 expression by Northern blot analysis (figure 6). (Tissue culture and assays were carried out by K. Bottomly, Yale)

5. *B10 and C11 are not expressed in peritoneal exudate lymphocytes*

To date, all active cytotoxic T cells have been positive for B10 and C11 expression, with the exception of some cloned lines generated from peritoneal exudate lymphocytes (PEL) (Zagury *et al.*, 1975). RNA prepared from four PEL-derived lines was generously provided by Y. Kaufman (Weiszman Institute) and analyzed by Northern blotting (figure 7). No detectable B10 or C11 mRNA was found in them (PEL #3, 4, 9 and 10) although a signal was apparent for the T cell lines MTL2.8.2 and D10. Hybridization with an actin

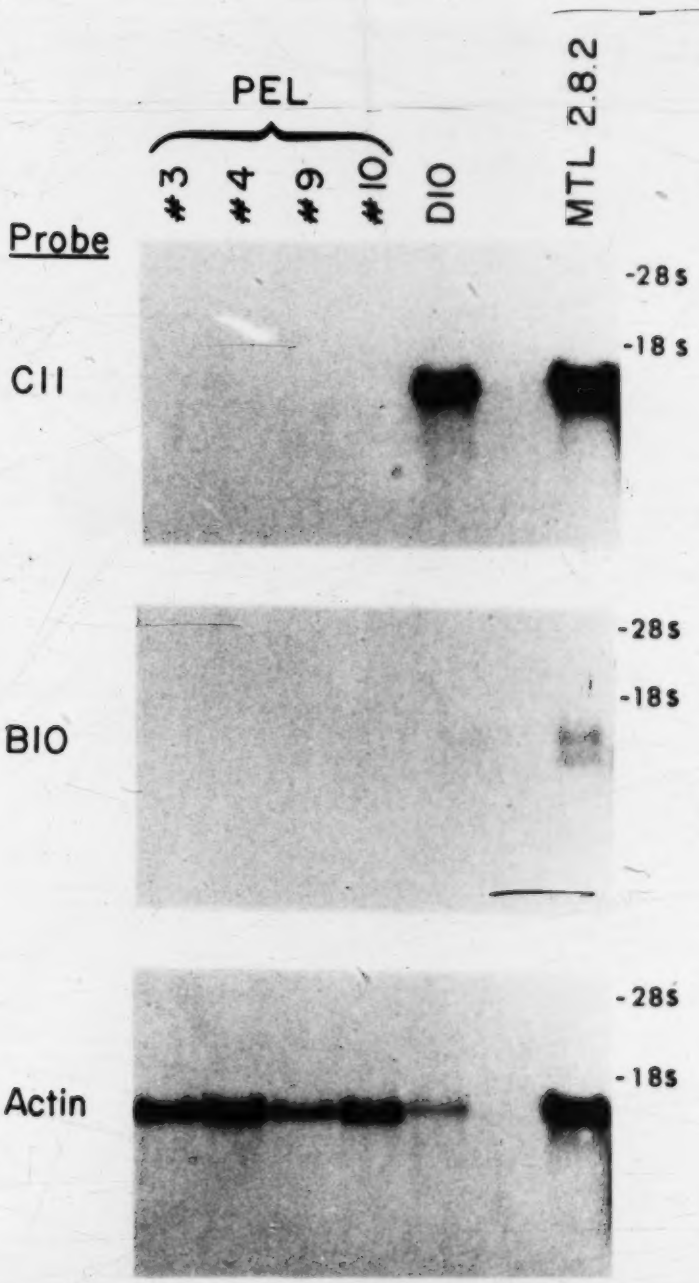

FIGURE IIIA.7 Analysis of B10 and C11 expression in peritoneal exudate lymphocytes (PEL). Total RNA from 4 cell lines derived from PEL, the D10 T cell line and the MTL2.8.2 CTL line was size-fractionated on a 0.8% formaldehyde-agarose gel and transferred by Northern blot. The blot was then probed in turn with nick-translated C11 cDNA insert, B10 cDNA insert and an actin gene probe.

probe showed that there was a comparable level of RNA from each of the cells. Interestingly, it was recently reported that although these cells have cytotoxic activity, the granules isolated from them do not have lytic activity (G. Berke, Intl. Cong. Immun.) and do not contain perforin. Consequently, these cells may kill their targets via a mechanism different from CTL which does not involve the granular-associated proteins such as serine proteases or perforin.

6. CsA and PMA do not inhibit C11 transcription

Cyclosporin A (CsA) is an immunosuppressive drug commonly used in organ transplantation to block rejection of the foreign tissue. At least part of its inhibition of a normal cellular immune response has been attributed to its ability to block production by helper T cells of lymphokines, including IL2, gamma-interferon and GM-CSF (Britten and Palacios, 1982; Bunges *et al*, 1981; Reem *et al*, 1983; Elliott *et al*, 1984). This blockage occurs at the level of transcription, that is, CsA selectively inhibits the transcription of a few specific mRNAs. In addition to its effects on helper T cells, it also appears to inhibit killer T cell function (Orosz *et al.*, 1982). When the antigen-dependent T cell clone, MTL21.9(I), was incubated with CsA after stimulation with alloantigen and exogenous IL2, the cytolytic activity of the cells was inhibited ~3-fold (measured by a ^{51}Cr-release assay; Shaw *et al.*, 1978). To test whether this inhibition of activity was associated with a decrease in the accumulation of C11 mRNA, MTL21.9(I) cells were stimulated with antigen and IL2 and after 2 days, half of the cells were incubated with 50 ng/ml CsA. RNA samples were prepared from the CsA-treated and untreated cells at 3 days post-antigen, run on a Northern blot and hybridized with C11 (figure 8A). Although the cytolytic activity of the cyclosporin-treated cells was inhibited, the level of C11 mRNA was approximately the same as in the untreated cells.

Another compound which can inhibit the lytic activity of memory CTL is phorbol myristate acetate (PMA; Orosz *et al.*, 1983). When a spleen cell culture which was

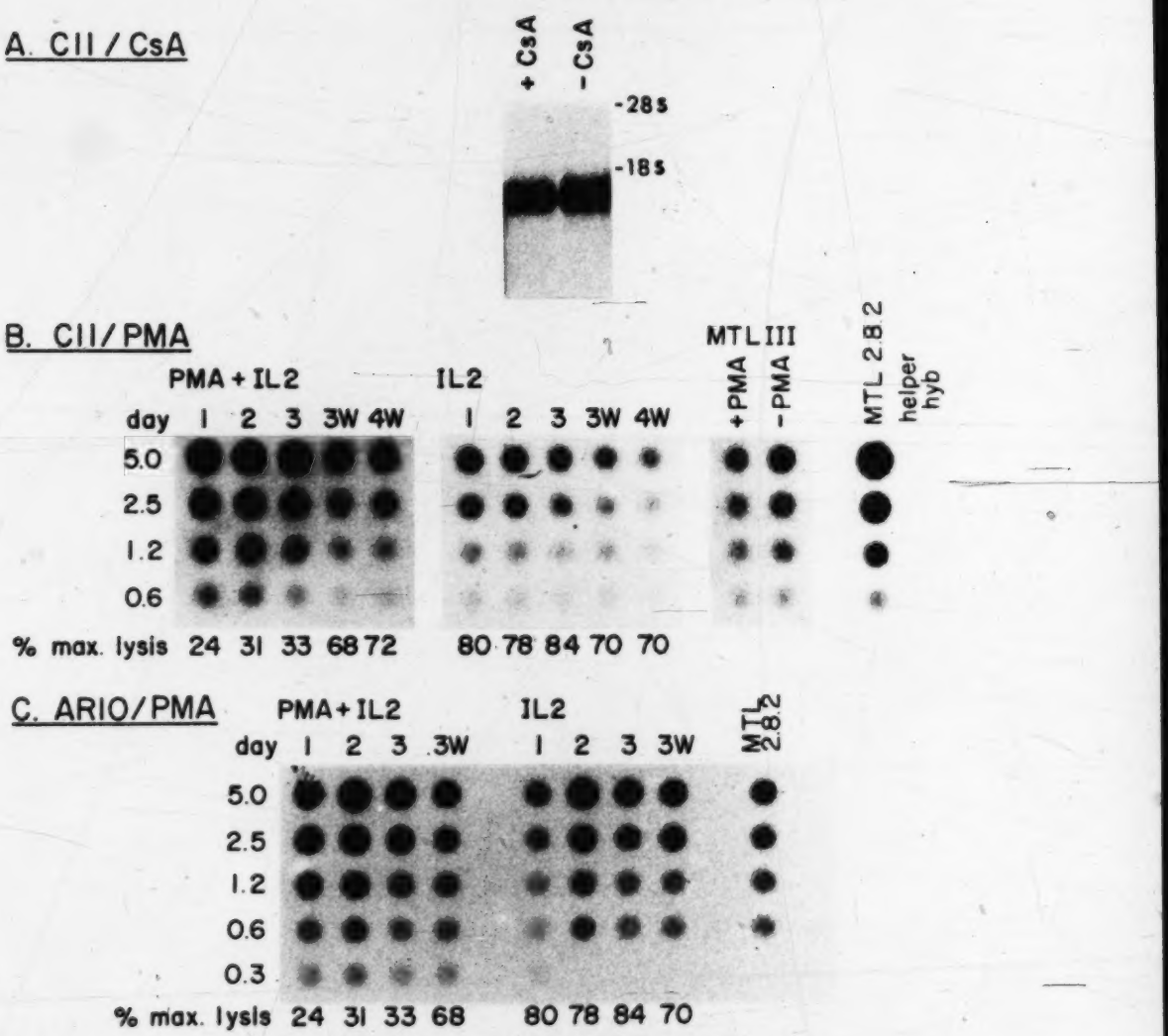

FIGURE IIIA.8 C11 expression in CsA- and PMA-treated cytotoxic cells. A. MTL21.9(I) cells were stimulated with antigen and 2 days later CsA (50 ng/ml) was added to half of the cells. A Northern blot was prepared with RNA from the treated (+CsA) and untreated cells (-CsA) and hybridized with nick-translated C11 cDNA insert. B. Spleen cells were stimulated with irradiated F(1) cells and IL2, and after 18 days, were re-stimulated with IL2 alone, in the presence (PMA+IL2) or absence (IL2) of PMA. RNA was prepared from cells at 1 to 4 days after stimulation. "3w" and "4w" indicate cell samples which were washed at day 2. RNA from the CTL line, MTL2.8.2(III), PMA-treated or untreated, MTL2.8.2(II) and a helper hybridoma was also included. The cytodot was hybridized with radioactively-labeled C11 cDNA. C. The same RNA samples were probed with nick-translated AR10, another CTL-specific cDNA clone. In (B) and (C), the days after stimulation are indicated along the top, and the number of cells X 10^{-5} along the side of the cytodots. The cytolytic activity is listed at the bottom of each lane.

originally stimulated with alloantigen and IL2 is re-stimulated with IL2, the cells proliferate and cytotoxic activity of the culture increases ~10-fold. If PMA is added together with the IL2, cell proliferation still occurs but there is only a ~3-fold increase in cytotoxic activity. To assay the effect of PMA on C11 expression, the long-term spleen cultures were stimulated with IL2 in the presence or absence of PMA (carried out by J. Hooten), and cell samples taken at days 1, 2 and 3 post-stimulation. At day 2, half of the IL2- and IL2+PMA-treated cells were washed and incubated with IL2 alone. This wash removes the effect of PMA-inhibition. Samples of the washed cells were taken at day 3 and day 4 (designated "3w" and "4w" in the figure). For each of the samples taken, the cells were assayed for cytotoxic activity and RNA was prepared for cytodot analysis (figure 8B; the corresponding cytolytic activity for each day is given underneath the cytodot). Also included on the cytodot were MTL2.8.2(III) cells incubated with or without PMA, and MTL2.8.2 and helper hybridoma cells as positive and negative controls, respectively. The cytodot was hybridized with C11, which gave a comparable signal intensity for the PMA-treated and untreated samples (figure 8B). This revealed that PMA does not inhibit the transcription of C11 mRNA, although the cytolytic activity is inhibited 3-fold. Washing the PMA-treated cells increased the cytolytic activity from 33% to 68% on day 3. This rise in activity, however, was not accompanied by a burst in C11 expression. The effect of PMA on the level of mRNA corresponding to another CTL-specific clone, AR10, was also assayed (figure 8C; Gershenfeld and Weissman, 1986). Transcription of AR10 mRNA is induced in an MLR similar to, but later than, C11 and B10. In the cultures tested here, AR10 mRNA increased and peaked at day 2. As for C11, this induction was not inhibited by PMA.

CTL activation and lysis is a complex multistep process, as discussed in chapter I. Initially the CTL recognizes a target cell and this recognition induces synthesis of the IL2 receptor subunit, allowing uptake of the lymphokine. The dual signal of antigen and IL2 activate the CTL to proliferate into a clonal population of cells capable of binding and lysing

target cells. Cytolysis occurs in three stages: target conjugation; a Ca^{2+}-dependent stage in which serine proteases are involved and a lethal signal is delivered to the target cell; and finally, release of the target cell, which subsequently lyses. The steps in CTL activation and lysis have largely been delineated by inhibiting the process at specific stages. For example, the lack of Ca^{2+} will not allow cytolysis to proceed beyond target conjugation and serine protease inhibitors block the subsequent stage in which the lethal signal is delivered to the target cell.

In the CsA-treatment of CTL described here, cytotoxicity was blocked without inhibiting cell proliferation. However, it was subsequently found that if CsA is added together with antigen and IL2 (at day 0), both proliferation and cytotoxic activity are completely inhibited (C. Havele, in preparation). CsA may therefore be acting to block antigen-triggering of intracellular events. This implies that proliferation and cytotoxic activity are sequentially induced by antigen. However, the fact that C11, which is induced late in the CTL response and may be involved in target cell lysis, is still expressed when cytotoxic activity is blocked suggests that induction of proliferation and induction of cytotoxicity are independent events.

PMA effectively inhibits the cytotoxic activity in the memory-type cells without blocking cell proliferation or C11 expression. However, PMA inhibition of cytotoxicity does not occur in an antigen+IL2-dependent response. On the contrary, it can substitute for antigen to stimulate the response. The difference in the effect of PMA in the two types of cells may be explained by an alternate pathway of activation used by the memory cell, which is antigen-independent and PMA-inhibited. However, PMA inhibition of this pathway does not block the proliferative response or C11 induction, suggesting that expression of some other molecule(s) involved in cytolysis is blocked.

The variability in the effects of CsA and PMA, depending on the time they are added and the cell's requirements for cell stimulation, suggest that rather than a simple sequence of events, CTL activation involves a network of signals. Some of the genes

which encode molecules that are induced in the CTL response have now been cloned, including the inducible subunit of the IL2 receptor, lymphotoxin, perforin and, of course, the serine proteases, CCPI and II and Hanukah Factor. These cDNAs, as well as antibodies against the protein products, can be used to monitor how inhibitors such as CsA and PMA are exerting their effects at the molecular level. In this way we should be able to refine our understanding of the series of events which occur during target recognition and lysis.

8. *Localization of CCPI to granules*

Synthetic peptides based on the C11 sequence were synthesized and used to generate antibodies against CCPI (RCB, in preparation). M. Redmond (U. of A.) used horseradish peroxidase-labelled antibody to localize CCPI to the characteristic granules of CTL. The antibodies detected a protein in the granules of the type II CTL clones, MTL2.8.2 and MTL2.2.1, which express C11, but not in EL4, S194 or CTL.L16 cells, which do not express C11. On Western blots, either of total cellular protein or of granular-associated proteins, the antibody bound to a protein at ~29 kD, both in non-reducing and reducing gels (RCB, in preparation). We conclude that this band and the protein localized to the granules is CCPI. Since the synthetic peptides correspond to regions of CCPI which are homologous to the B10-encoded protein, CCPII, the immuno-staining may include CCPII as well as CCPI.

CTL granules are believed to contain the effector molecules for CTL-mediated lysis. Isolated granules from CTL contain perforin and serine proteases and are capable of non-specific target cell lysis (Podack and Konigsberg, 1984; Masson *et al.*, 1986a). Recently, one of these granule-associated proteases was purified to homogeneity and sequenced (Masson *et. al.* 1986b). The amino acid sequence determined is identical to that predicted for Hanukah Factor, based on the cDNA sequence of AR10 (Gershenfeld and Weissman, 1986). This protein runs at 60 kD on a non-reducing gel and 35 kD on a reducing gel. At

least one other serine protease has been detected in granules by diisopropylflourophosphate (DFP)-binding (Young *et al.* , 1986). It is reported to run at 29 kD on both reducing and non-reducing gels and seems to have an unusual substrate specificity. Therefore it quite likely is CCPI. The existence of CCPI as a monomer wasn't expected, since an uneven number of Cys-residues occurred in the derived amino acid sequence. This lead to the suggestion that the Cys at position 74, which has no counterpart in other known serine proteases, is linked by a disulphide bond to another chain (Reid, 1986). CCPI was therefore predicted to correspond to the serine protease homo- or heterodimer which ran at 60 kD on a non-reducing gel and 35 kD on a reducing gel (now known to be Hanukah Factor).

The CTL-L16 line is an IL2-dependent clone which has lost its cytotoxic activity even when incubated with the mitogen, ConA (C. Havele, personal communication). RNA was prepared from CTL-L16, with and without a 16 hr incubation with ConA, and used to prepare a Northern blot. When probed with B10 and C11, no B10 or C11 mRNA was detected, either in the samples from unstimulated or mitogen-treated cells. The lack of B10 and C11 expression may be the cause of the loss of CTL-L16 cytotoxic activity, although it is possible that other molecules involved in cytolysis are also not expressed. CTL-L16 cells have a high number of the characteristic dense T_K granules. However, when the granule-associated proteins from CTL-L16 were analyzed on a Western blot by probing with the anti-CCPI antibody, no signal was detected. This absence of CCPI in the CTL-L16 granules agrees with the lack of C11 mRNA in the cells.

8. *Other related DNA clones*

(a) D12

A cDNA library was constructed using the method of Land *et al.* (1981) to generate double-stranded DNA (dscDNA) from MTL2.8.2 mRNA. The dscDNA was ligated into the pUC13 plasmid vector via EcoRI linkers (Goodman and MacDonald, 1979) and the

recombinant DNA was used to transform *E. coli* JM83 (Maniatis *et al.*, 1982). This library was then screened with B10 and C11 insert DNA in order to isolate full-length clones. A cDNA clone which cross-hybridized strongly with B10 and C11 was identified and found to have an insert of 830 bp. On Northern blots, this clone hybridized to an mRNA band of ~1000 ntd, the same size as the B10 mRNA. However sequence analysis revealed that this clone is not B10, but a third member of this highly related gene family.

The D12 insert was subcloned into M13 and preliminary sequence data was obtained (figure 9; Sanger *et al.*, 1980). Comparison of the D12 sequence to that of C11 revealed a 63% homology at the nucleotide level, which accounts for the cross-reactivity of the clones. The putative translation start site was identified by alignment to the C11 sequence, although to align it throughout the C11 translated sequence, the reading frame must change. The sequence data must therefore be verified to determine if D12 does encode a homologous protein to CCPI.

Assuming further sequence data will yield an open reading frame encoding a CCPI-like protein, the D12 cDNA clone includes a 166 bp 5'-untranslated sequence, but does not extend to the 3'-end of the translated sequence. A potential ribosome binding site, CCUUCC (Hagenbuchle *et al.*, 1978) is located 13 ntd upstream of the putative start codon. The D12-encoded protein would have a hydrophobic signal sequence, with an Ala-Gly cleavage site 4 amino acids before the Ile-Ile which is predicted to form the amino-terminus of the mature form of CCPI (chapter III; Lobe *et al.*, 1986b). This implies the sequence of the activation peptide would be Gly-Ala-Glu-Glu, compared to the CCPI predicted propeptide of Gly-Glu. Most importantly the homology to C11 suggests that the D12 transcript would also encode a serine protease related to CCPI and II. Of the catalytic triad, the His and Asp are both present in the same locations as in CCPI. Because the D12 sequence stops short of the 3'-end of the translated region, we do not yet know if the Ser is present in the putative D12 protein product, or if it would possess the unusual substrate specificity predicted for the C11 protein product, CCPI.

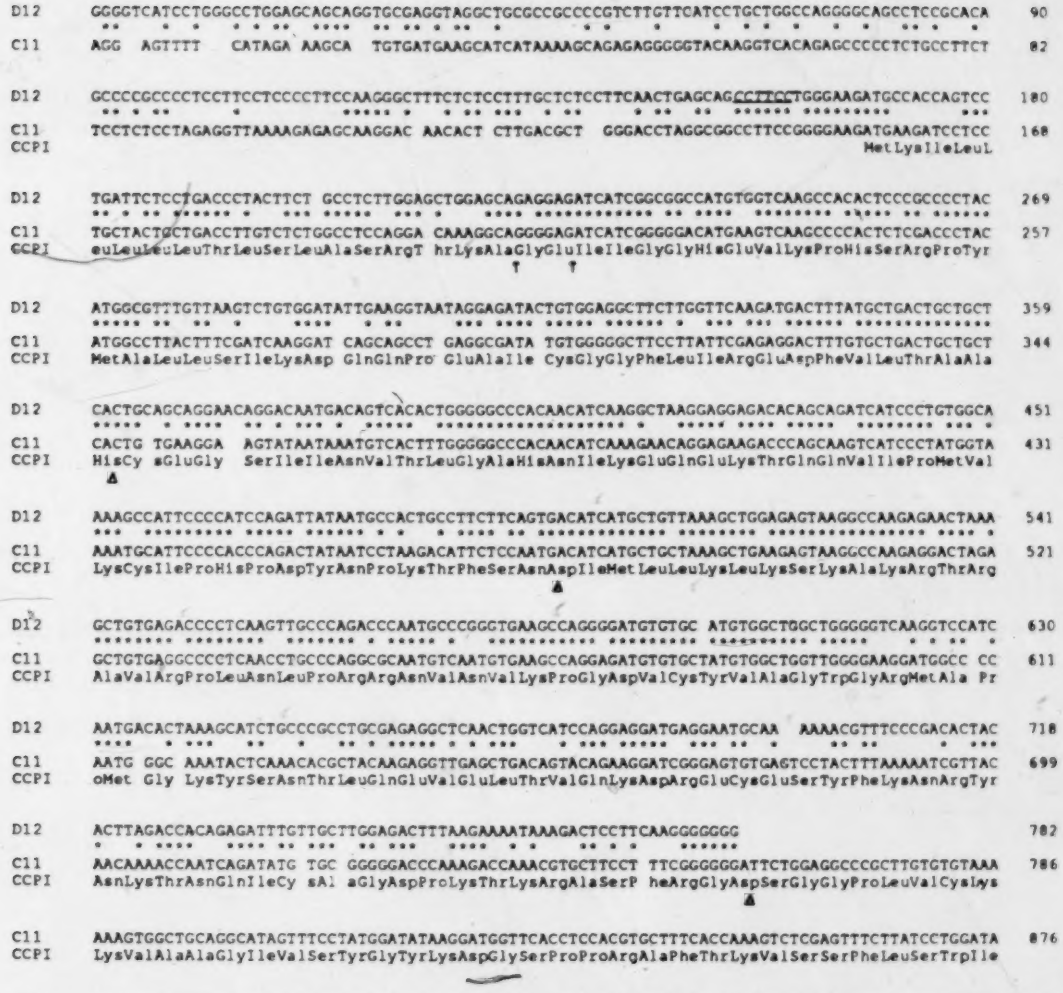

FIGURE IIIA.9 Nucleotide sequence comparison of the D12 and C11 cDNA inserts. The D12 insert was re-cloned in M13 and sequenced by the dideoxy method (Sanger *et al.*, 1975). The D12 sequence is shown optimally aligned with the C11 sequence (chapter III, Lobe *et al.* , 1986b) with asterisks designating nucleotide identities. The C11-translated product, CCPI, is shown on the bottom line. The putative ribosome binding site in the D12 sequence is underlined. The His, Asp and Ser residues of the catalytic triad are marked with arrowheads, and the probable cleavage sites of the signal and activation peptides are indicated with arrows.

When used to probe the same cell panel cytodot as B10 and C11, D12 was also found to be expressed only in T cells with cytolytic activity. It was expressed in an IL2-dependent, antigen-independent (type II) CTL line, MTL2.8.2(II), and an IL2-independent variant, MTL2.8.2(III) (figure 10). The D12 transcript was not detected in brain, liver, thymus, splenic NK or helper hybridoma cells (figure 10). By Northern blot analysis, however, the ~1000 ntd D12 transcript did not appear to be expressed in ConA-stimulated spleen cells or in the antigen-dependent CTL line, MTL21.9(I) (figure 2). The faint higher MW band seen for MTL21.9(I)+antigen is probably due to cross-hybridization to the higher molecular weight C11 transcript, as is the signal for the long-term MLR+IL2 in figure 10. These results have been confirmed in three other experiments in which cells from an MLR or from antigen-stimulated type I CTL were tested for D12 expression. The physiological significance of a high level of expression of D12 in CTL lines which have become antigen-independent and not in normal CTL is not known. Unlike the antigen-dependent T_K cells, which do not have a detectable level of D12 mRNA, the antigen-dependent $T_{H/K}$ cells have a level of D12 mRNA approximately equal to B10 mRNA (section B.3; figure 4A and 5). Possibly, then, D12 corresponds to an mRNA whose physiological role is in this recently identified subset of T cells, $T_{H/K}$. On the Northern blot, the helper cell lines (CH2.4 and EL4), RI lymphoma, S194 myeloma and splenic B cells were all negative for D12 (figure 2).

b) gN1

Another clone which cross-hybridized with C11 was identified while screening for a genomic C11 clone in a lambda charon 4A phage library. This genomic DNA clone, designated gN1, has an insert of ~10 kb. The restriction map of this clone, however, did not correspond to the map of the C11 gene deduced from probing total genomic DNA. When used to probe the same cell-panel cytodot as the other clones, it was found that unlike B10, C11 or D12, this clone appears to be NK-specific (figure 10). A strong

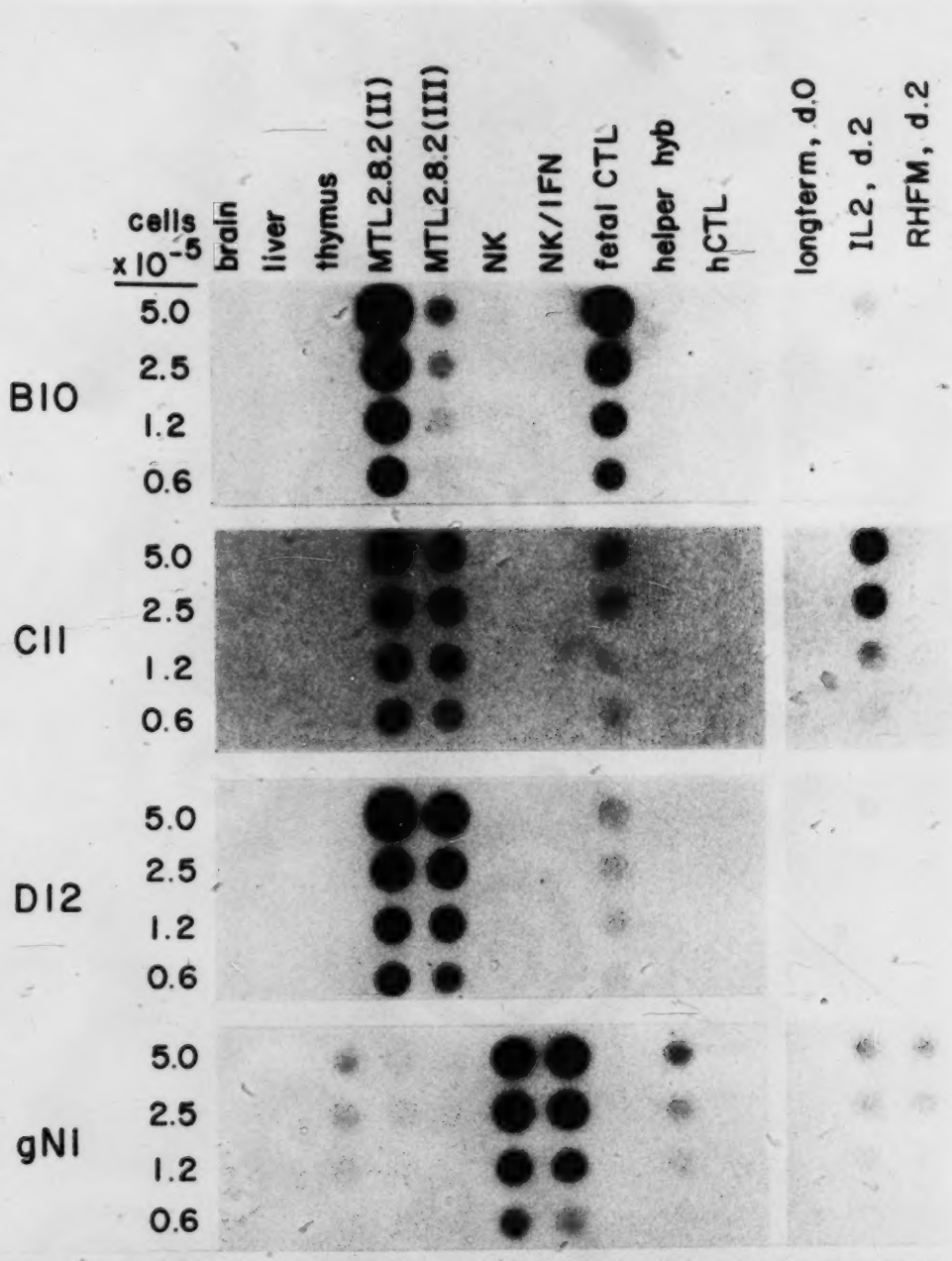

FIGURE IIIA.10 Tissue specificity of D12 and gN1 expression. The cytodot described in figure IIIA.2 was washed and re-probed with nick-translated D12 and gN1 DNA (lower panels). The results already presented for the C11 and B10 probe are shown in the upper 2 panels for comparison.

hybridization signal was seen for splenic NK cells, with or without pre-incubation with interferon. A much lower level of expression was seen in thymus cells, the helper hybridoma, the long-term MLR culture (with either IL2 addition or a change of media) and type II MTL2.8.2 (figure 10). From the cross-hybridization of gN1 to C11, B10 and D12, it is apparently a member of this set of related sequences, perhaps also encoding a serine protease. Its NK-specific expression is intriguing, since it has been suggested that CTL and NK cells lyse their targets by a similar mechanism (Henkart *et al.*, 1984; Quan *et al.*, 1982). Assuming that the C11, B10 and gN1 protein products are involved in cytolysis, the two types of cells may employ different but very homologous proteins to carry out their functions.

C. CONCLUSIONS

All T cell lines with cytotoxic activity or cultures which contain active cytotoxic T cells thus far tested express the C11 and B10 transcripts. D12 expression is also associated with cytotoxic T cells, but doesn't appear to be required for cytotoxic activity. In all cases, the level of C11 and B10 mRNA increased when antigen-dependent cytotoxic T cell lines or spleen cell cultures were activated by antigen or ConA, with the peak of induction preceding the peak of cytotoxic activity. In spleen cells stimulated with alloantigen, B10 and C11 mRNA levels did not peak until 2 to 3 days post-stimulation, approximately one day prior to the peak of cytotoxicity. When a long-term MLR reaction was re-stimulated and cytotoxicity peaked by day 1, C11 and B10 induction also occured by that time. In a response in which a cloned CTL line was re-stimulated and its activity returned by 9 hours to 2 days (depending on the cell line), induction of C11 and B10 expression always occurred just ahead of the increase in cytotoxicity. In alloantigen-activated spleen, C11 induction occured first, followed by B10 ~12 hrs later, and cytotoxicity 12 hrs after that, so the two mRNAs may be sequentially regulated.

As discussed in the introduction, two signals are required to activate a pCTL: antigen and IL2. The initial signal of antigen causes a transient burst of transcription of a few genes, including an IL2 receptor subunit, c-myc and MHC molecules. The increase in C11 and B10 mRNA, however, appears to occur later than this initial response. Their expression may be induced by secondary messengers later on in the series of events which may ultimately lead to target cell lysis. The molecules initially synthesized are probably involved in propagating the antigen recognition signal, whereas the molecules which are subsequently induced are involved in the lytic process itself. Indeed, the kinetics of induction of C11 are similar to those of LT mRNA in a LT-secreting T cell line. However, although C11 and LT are both induced and at about the same time, their induction appears to be mediated by different signals. Cyclohexamide treatment during antigen activation of $T_{H/K}$ cells revealed that C11 induction is mediated by a regulator which can be blocked by cyclohexamide, and LT mRNA induction is probably mediated by a different molecule, which is not blocked by cyclohexamide.

Several $T_{H/K}$ cell lines were assayed for B10 and C11 expression, BLT-esterase activity and cytotoxic activity. In all of the lines with cytotoxic activity, C11 and B10 were expressed. There was no correlation with BLT-esterase activity. However, the BLT-cleavage is an assay for trypsin-like activity, which probably corresponds to another CTL serine protease, Hanukah factor. CCPI and II are predicted to have an unusual substrate specificity, and therefore not expected to be detected in the BLT assay. C11 and B10 expression correlated with cytotoxic activity but not with BLT-esterase activity. Of note, BLT-esterase activity did not correlate with cytotoxicity.

Cytotoxic T cell lines which have been generated from peritoneal exudate lymphocytes have granules, but, unlike those from other CTL lines, the granules isolated from PEL-derived lines do not have cytolytic activity or perforin. Therefore they must employ some other mechanism to lyse their target cells. By Northern blot analysis, these

cells do not express C11 or B10. Thus, the only cytotoxic T cells found not to express B10 and C11 also seem to employ an exceptional mechanism for target cell lysis.

Although cytotoxic activity can be blocked by CsA and PMA, neither compound inhibits the corresponding increase in C11 mRNA. These compounds may either block other molecules necessary for cytolysis or block at a later stage of the process than induction of C11 transcription.

Two other members of the B10/C11 gene family were identified by cross-hybridization to C11. The first, D12, has so far only been found to be expressed in antigen-independent T_K cell lines and antigen-dependent $T_{H/K}$ cell lines. The lack of detectable D12 mRNA in antigen-stimulated spleen cultures or antigen-dependent CTL leaves open the possibility that the D12 gene is not normally expressed, but is aberrantly activated when T_K cells are grown in culture. Preliminary sequence data revealed that D12 may encode a protease homologous to CCPI.

The other C11-related sequence is the genomic clone, gN1. This clone is very homologous to C11 (by cross-hybridization), but unlike the other members of this gene family, gN1 is an NK-specific clone. C11 and B10 mRNAs have not been detected in splenic NK cells, although NK cells are thought to lyse their targets in a similar, granule-mediated mechanism to CTL. gN1 might therefore be the C11-equivalent in NK cells.

Localization of CCPI to CTL granules provides further support that the proteins are involved in the cytolytic event. Granules have previously been implicated in the mechanism of T cell-mediated lysis (Podack and Konigsberg, 1984). A model of granule-mediated killing is presented in figure 11. Upon interaction of the CTL with its target, cytoplasmic granules polarize to the point of contact between the two cells (Yanelli *et al.*, 1986). By fusing with the cytoplasmic membrane the granular contents are released into the contact space between the cells. One of these proteins contained inside the granules, perforin, is capable of forming a polymerized complex, containing hydrophobic and hydrophilic domains, which can be inserted into the target cell membrane and thus create a

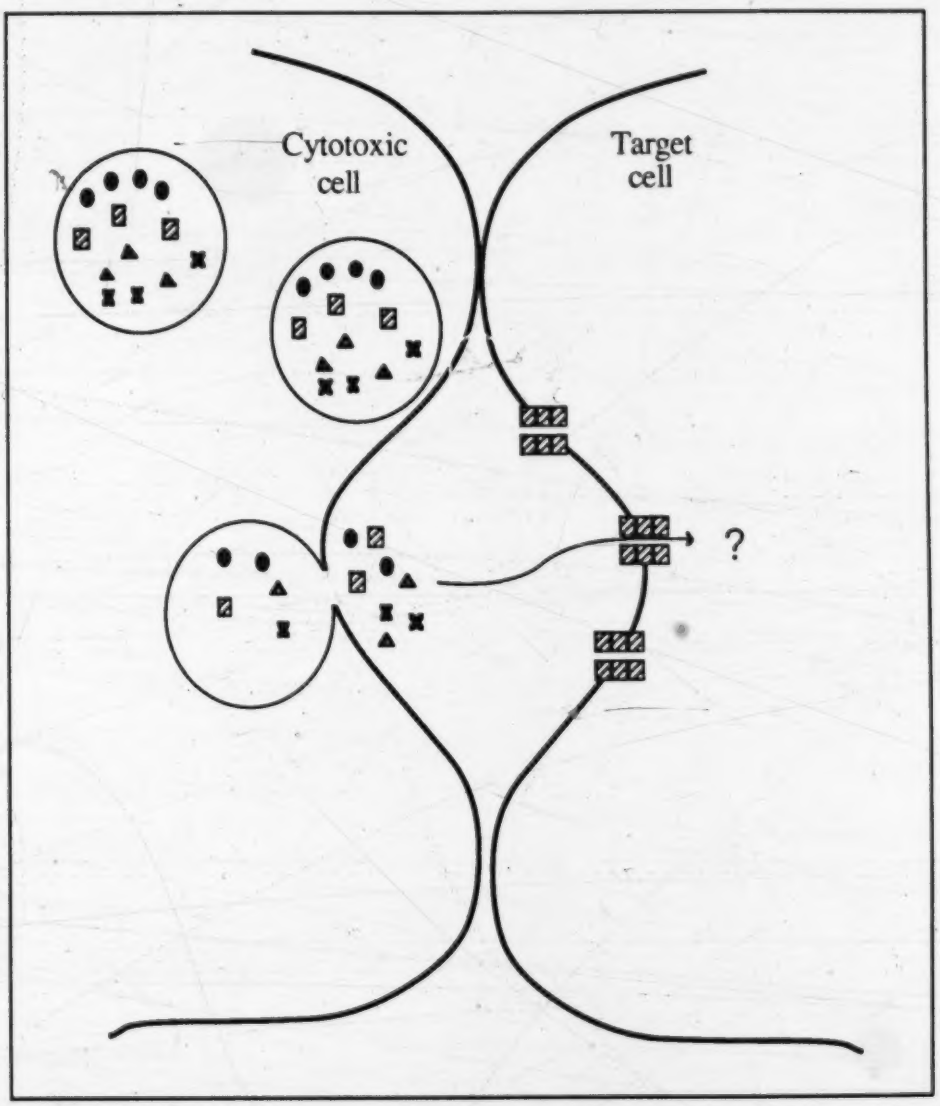

FIGURE IIIA.11 Model of CTL-granule involvement in killing. The granules of a cytotoxic cell, which has come into contact with a target cell, polarize and fuse to the CTL-target contact region. The granule-associated proteins can then be released into the intercellular space. Poly-perforin complexes are shown inserted into the target cell membrane, creating a trans-membrane channel. Other granular proteins may then pass into the target cell to induce destruction.

transmembrane channel (Dennert and Podack, 1983). The "lytic-molecules" could then pass into the target cell and induce its destruction.

The nature of the lethal effector molecules is not known, but presumably they are contained within the granules. The fact that CCP1 is located in the granules of cytolytically active T cells suggests that it may play a key role in the lytic mechanism. Indeed inhibitors of serine proteases have been shown to block target cell lysis even when introduced late in the process (Redelman and Hudig, 1983). The granules in CTL are acidic, so it is unlikely that CCP1 would be active at this pH (Masson *et al.*, 1986a). Therefore it is concluded that the proteases are active either during or after exocytosis. They could play a role in the degranulation process itself by attacking cell membrane proteins or, in a similar manner, facilitate polyperforin insertion in the target cell membrane. The analogy with complement-mediated lysis would suggest that the esterases may be involved in activating or assembly of the lytic molecules. Finally, it is possible that one or more of the proteases could pass through the transmembrane channels and, finding their substrate inside the target cell, initiate a chain of events resulting in cell death.

Although the precise functions of the B10- and C11-encoded proteins are not yet known, the results presented in this chapte. demonstrate that they are specifically expressed in CTL, their expression correlates with cytolytic activity and they are located in the CTL granules. Together, this strongly suggests that they play a important role in the lytic mechanism of CTL.

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Chapter IV

Organization and Regulation of the C11 and B10 Genes

A. INTRODUCTION

Cytotoxic T lymphocytes (CTL) belong to the effector subset of thymus-derived (T) cells. After differentiation in the thymus, these cells circulate in the periphery as precursor CTL (Scollay *et al.*, 1984). If they encounter cells bearing foreign antigens they become activated killer cells capable of binding to a target cell and causing it to lyse (Nabholz and MacDonald, 1983). Because of their role in defending the body against invasions such as viral infections or tumor formation, an understanding of how CTL function has important biological implications and clinical applications.

To examine the mechanism by which a CTL delivers a lytic signal to a target cell, we previously isolated cDNA clones representing mRNAs specifically expressed in CTLs (Lobe *et al* ., 1986a). Sequence analysis of two of these clones (C11 and B10) revealed that they were remarkably homologous to each other, with 80% identity at the nucleotide level. The full-length C11 clone encodes a protein of predicted MW25,319, designated cytotoxic cell protein (CCPI). This protein appears to be a serine protease, since the amino acid residues which form the catalytic triad of the serine protease active site and the sequences neighbouring these residues, which are highly conserved among proteases, are present in CCPI (Lobe *et al.*, 1986b). From the partial sequence of the B10 clone, it also appears to encode a serine protease, referred to as CCPII.

The predicted C11 protein product has 12 residues at the N-terminus which are highly hydrophobic, suggesting that this is part of a signal sequence to direct secretion or intracellular organelle location (Lobe *et al.*, 1986b). Indeed, using antibodies generated against a synthetic peptide derived from the CCPI sequence, the protein has been localized in the cytoplasmic granules (RCB, submitted). Following the signal sequence is a di-peptide, Gly-Glu, believed to be the activation peptide, as proposed for cathepsin G

(Salveson *et al.*, 1987). This is based on the observations that the Ala-Gly at that position is the likely cleavage site for signal peptidase and the amino-terminus of the mature enzyme probably starts with Ile-Ile, as it does for cathepsin G and rat mast cell protease type II. Such a short activation peptide of only two amino acid residues would be novel for a serine protease. By analogy to other serine proteases, the activation peptide is cleaved from the pro-enzyme to convert the inactive zymogen to the active form of CCPI (Neurath and Walsh,1976; Salvesen *et al.*, 1987).

CCPI is very homologous with rat mast cell protease typeII (RMCPII). This was particularly interesting, as RMCPII possesses a number of unusual structural features which indicate that it has a substrate specificity quite different from classical serine proteases (Woodbury *et al.* , 1978; Woodbury and Neurath, (1980). CCPI shares several of these features and in addition has other unique changes which alter the environment in the active site pocket (Lobe *et al.*, 1986b), suggesting that it too has unusual substrate specificity.

The level of expression of the mRNAs corresponding to B10 and C11 correlated with cytotoxic activity (Lobe *et al.*, 1986a). The maximum expression preceded the peak of cytotoxic activity in an *in vitro* allogeneic or mitogen induced response, by 12 to 24 hr. Together with the sequence information, this suggested that the protein products may well play an important role in mediating the killer cell function. The time course of expression together with the sequence data raises the possibility of a protease cascade mechanism of activation, analogous to the activation of the complement components (Reid and Porter, 1981). Alternatively they may themselves be toxic molecules which are directly involved in the destruction of the target cell.

The dramatic increase in the levels of the CTL-specific mRNAs upon cell activation posed the intriguing question of how their expression is regulated. This regulation could occur at the level of gene transcription, post-transcriptional processing or mRNA degradation (Darnell, 1982). We have used a nuclear run-off transcription assay to

determine if the increase in mRNA level is due to an increase in initiation of transcription. As a first step to characterizing the genes corresponding to B10 and C11, genomic clones have been isolated and mapped by restriction enzyme analysis. Some information on the exon/intron organization of the C11 gene was also obtained. This was of interest, since the gene organization of many serine proteases has been determined, and on that basis they can be classified into distinct evolutionary families. The C11 and B10 genes appear to be members of the same multigene family, which may also include D12, gN1 (appendix to chapter III), RMCP II (Lobe *et al.*, 1986b) and cathepsin G (Salvesen *et al.*, 1987). Therefore a characterization of how the genes are organized should provide information on how similar these genes are to each other, and how they relate to the other serine protease families. Possible regulatory sites of each gene have been identified by DNase I hypersensitive site mapping. The correlation of the hypersensitive sites with activity of the genes was investigated by assaying a variety of cells which differ in their state of expression of the B10 and C11 mRNAs.

B. MATERIALS AND METHODS

1. *Cell Culture*

The cytotoxic T cell lines MTL 2.8.2 and MTL 2.2.1 were generated from CBA/J mice as described (Bleackley *et al.*, 1982). The CTL.L16 line, kindly provided by Ann Cook (Middlesex Hospital, London), is a cytotoxic T cell line, but since being isolated has lost its cytolytic activity. EL4.E1 is an IL2-producing variant of EL4 (Farrar *et al.*, 1980) and S194 is a myeloma cell line. All cells were maintained in RPMI-1640 supplemented with 10% fetal bovine serum and 10^{-4} M 2-mercaptoethanol (RHFM). For the MTL and CTL.L16 lines, 10 U/ml IL2 was also included. ConA stimulation of the CTL.L16 cells

was carried out at 5 ug/ml for 16 hrs prior to harvest. Thymus cells were obtained from 11-week old CBA/J mice.

2. *DNA Preparation*

Phage or plasmid DNA was purified by the plate lysis or rapid alkaline lysis methods respectively (Maniatis *et al.*, 1982). To prepare genomic DNA, 10^7 cells/ml were lysed in 0.5%SDS, the solution was adjusted to 100 ug/ml proteinase K and incubated at 37 C overnight. Following phenol/chloroform extractions, the solution was adjusted to 0.3 M NaOAc and 67% ethanol. High molecular weight DNA was spooled out on a pasteur pipette, air dried, rinsed with 70% ethanol and dissolved in 10 mM Tris-HCl pH8/1 mM EDTA at 4 C.

Restriction enzyme digests were carried out in 1X Core Buffer (BRL) at 37 C, either overnight (phage and genomic DNA) or from 1 to 4 hrs (plasmid DNA). Digested DNA was then electrophoresed on agarose gels and analyzed by Southern blotting (Southern, 1975). Alternatively, insert DNA was isolated by polyacrylamide gel electrophoresis and purifed by the crush-soak method (Shleif and Wensink, 1981).

3. *Molecular Probes and Hybridizations*

The DNA probes used were inserts or subfragments from the CTL-specific clones B10 and C11 (Lobe *et al.* , 1986b), purified by polyacrylamide gel electrophoresis and the crush-soak method (Schleif and Wensink, 1981). Insert from a rabbit beta-globin gene clone (Grosveld, 1981), which is homologous and cross-hybridizes to the mouse sequence (van Ooyen *et al.*, 1979), was also used as probe. The DNA probes were nick-translated using a BRL nick-translation kit or oligo-labelled (Feinberg and Vogelstein, 1983) to 1 to 5 x 10^8 cpm/ug. A synthetic oligonucleotide was purchased from The Regional DNA Synthesis Laboratory, Calgary, and corresponds to the sequence 5'-CCTCTCTGCTTTTATGATGC-3'. This 20-mer is homologous to a sequence 106 ntd

upstream from the start site in the C11 mRNA, and was kinase-labelled to 1 x 10^8 cpm/ug (Maniatis *et al.*, 1982) and used as a probe.

Blots were pre-hybridized in 50% formamide, 20 mM phosphate buffer pH6.8, 2 mM pyrophospate, 100 uM ATP, 5XDenhardts, 5XSSC, 100 ug/ml salmon sperm DNA, 0.1% SDS and 2.5 mM EDTA, at 47 C for 2 to 15 hrs. Hybridization was carried out in the same buffer, with 1 to 5 x 10^6 cpm/ml of DNA probe. After 15 hrs at 47 C, blots were washed in 0.1XSET-0.1%SDS for 60' at 65 C with 3 changes of buffer. The filters were then exposed to X-ray film using an intensifying screen.

4. *Screening of Genomic Libraries*

The genomic B10 clone was isolated from a genomic library prepared from CBA/J mouse liver DNA in the lambda charon4A vector (Drs. R.C.Bleackley and J. Bell, University of Alberta). Two million recombinants, propagated in *E. coli* NEM259, were screened in duplicate at 2 x 10^5 plaques per plate. Another murine genomic DNA library, generously provided by Mark Davis (Stanford), was used to isolate the genomic C11 clone. The library contained CBA/J mouse liver DNA in the vector lambdaJ1, and was grown in *E. coli* DL191. A total of 2 x 10^6 plaques were screened in duplicate. Plaques were lifted onto nitrocellulose filters, denatured in 0.5 M NaOH/1.5 M NaCl and neutralized in 1 M Tris-HCl pH7/1.5 M NaCl. The filters were allowed to air-dry, then baked at 80 C for 1.5 hrs in a vacuum oven. They were then hybridized with insert DNA from B10 or C11, washed and exposed to X-ray film, as described above.

5. *Nuclei Preparations and DNase I Treatment*

Nuclei were prepared by a method similar to that described by Enver *et al.* (1985). Harvested cells, 1 to 2 x 10^7, were banded on a Ficoll gradient, then rinsed in cold PBS and transferred to 15 ml snap-cap tubes. The cells were resuspended in 1 to 2 ml ice-cold lysis buffer (50% glycerol, 50 mM Tris-HCl pH7.9, 100 mM KCl, 5 mM $MgCl_2$, 0.05%

NP40, 200 mM 2-mercaptoethanol) and left on ice for 10' to lyse. Nuclei were pelleted by centrifugation at 5500 rpm in a Sorvall HB4 at -5 C, rinsed in ice-cold Buffer A (100 mM NaCl, 50 mM Tris-HCl pH8.0, 5 mM $MgCl_2$, 0.1 mM PMSF) and re-pelleted at 5500 rpm for 3' at 0 C. The nuclear pellet was resuspended in 2 ml ice cold Buffer A + 0.5 mM Ca^{2+}, except CTL.L16 nuclei which were resuspended in 1.2 ml. Aliquots of 180 ul were incubated at 37 C for 15' with 20 ul of DNase I, diluted to various concentrations in buffer A + Ca^{2+}. Digestion was terminated by the addition of EDTA to 25 mM. RNase was added to 20 ug/ml and the incubation was continued for 30' at 37 C. Proteinase K and SDS were then added to 50 ug/ml and 0.25% respectively, and the reactions left overnight at 37 C. Following extraction with phenol and chloroform, the genomic DNA was ethanol precipitated and resuspended in 100 ul 10 mMTris-HCl/1 mM EDTA, pH8. Samples were then digested with BamH1 overnight and electrophoresed on 0.8% agarose gels at 30 V for 20 to 24 hr (usually one quarter or one half of each sample was used per lane). The DNA was transferred to nitrocellulose (Southern, 1975) or hybond nylon membranes (Amersham) and hybridized.

C. RESULTS

1. *Restriction map of B10 and C11 genes*

The C11 and B10 genes were initially characterized by restriction enzyme mapping. Genomic DNA was digested with various restriction enzymes, sized by agarose gel electrophoresis and transferred to nylon membranes. The southern blots were then hybridized successively with the C11 insert, the BamH1, PstI and Sau3A sub-fragments of the C11 insert, the B10 insert and PstI sub-fragments of the B10 insert (see cDNA maps, figure 1). The autoradiographs of one of the Southerns probed with a C11 5' fragment and with the B10 insert are shown in figures 2A and B, respectively. By compiling the data from each of the probes, together with data from the genomic clones described below, a restriction map was obtained for the B10 and C11 genes (figure 3).

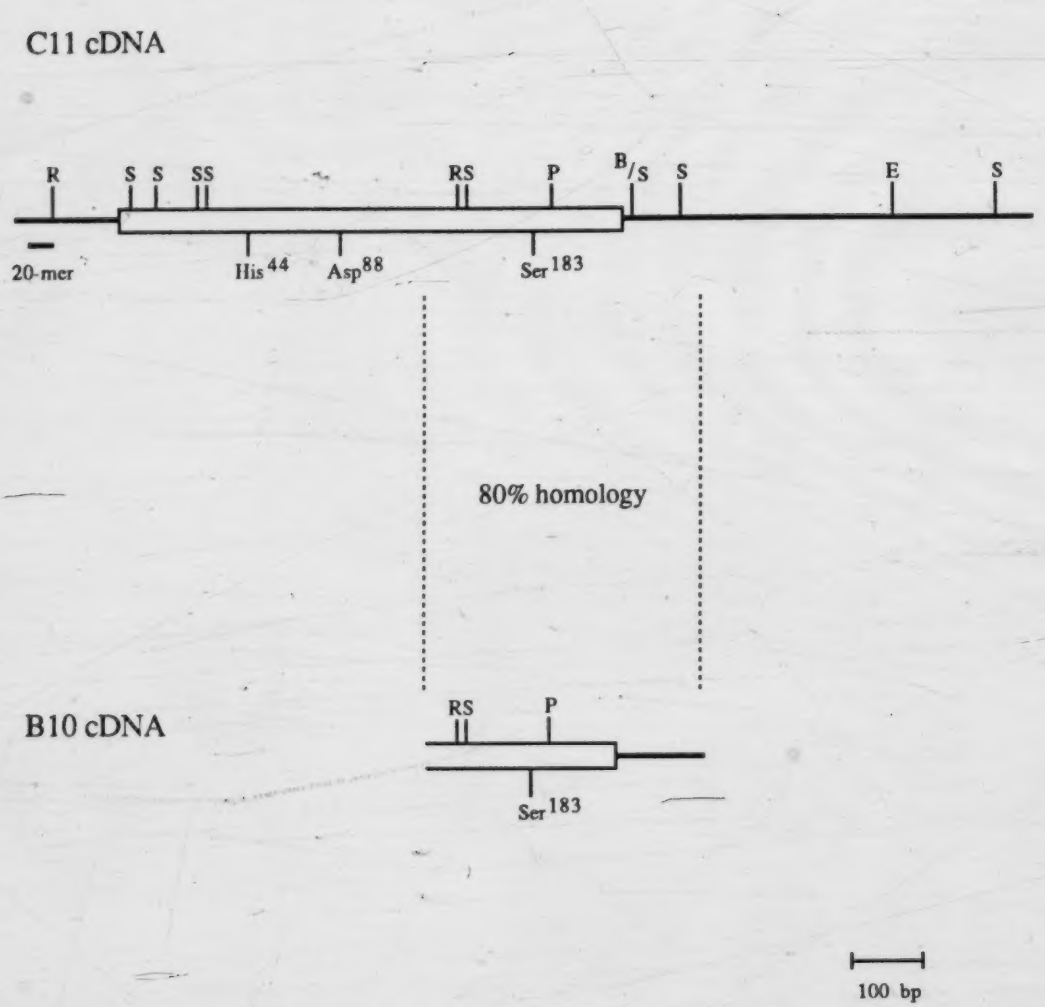


FIGURE IV-1. Restriction enzyme maps of C11 and B10 cDNAs. The map of the restriction enzyme sites used to isolate subfragments of the cDNA and to map the genomic clones is shown, based on the nucleotide sequence (chapter III; Lobe et al., 1986b). The translated region is shown as a box, with the position of the codons for the catalytic triad residues (His, Asp and Ser) shown underneath. The location in C11 to which the synthetic 20-mer is complementary (100 ntd upstream from the translation initiation site) is also shown. Restriction enzyme sites marked are RsaI (R), Sau3A (S), PstI (P), BamHI (B) and EcoRI (E).

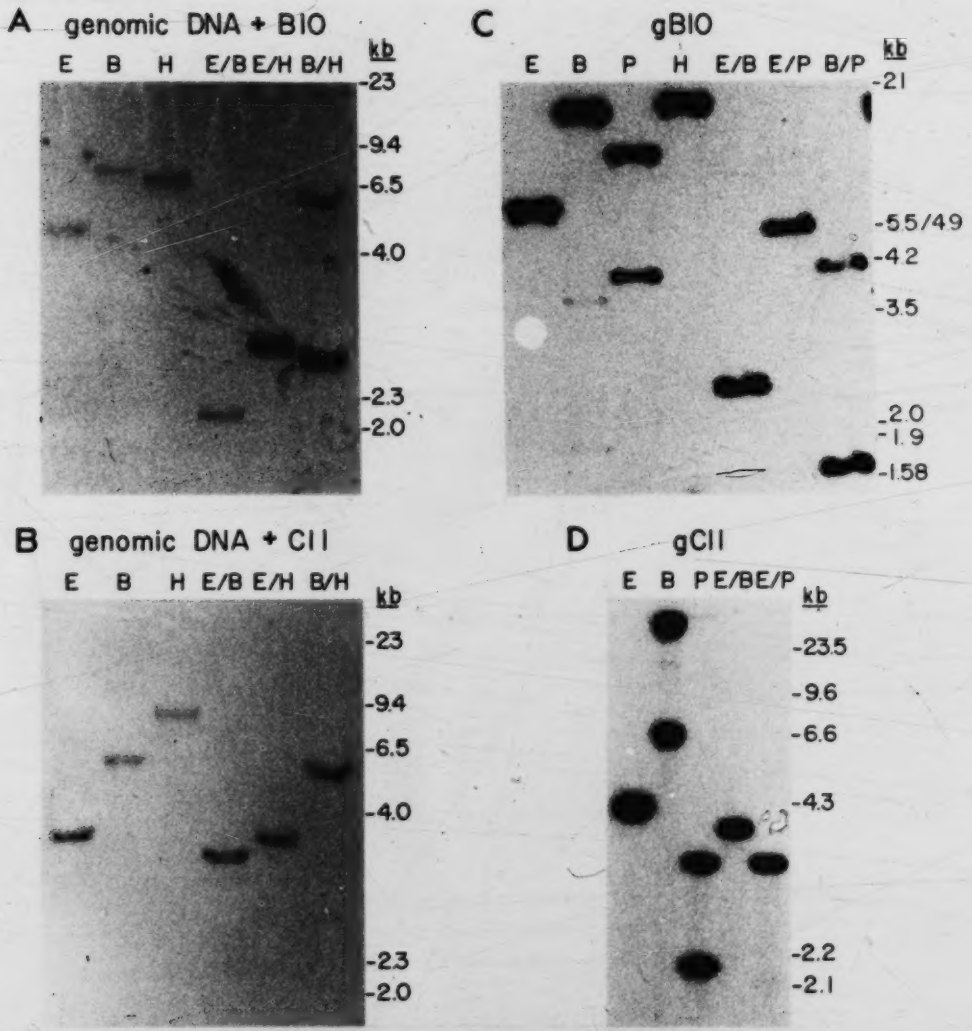
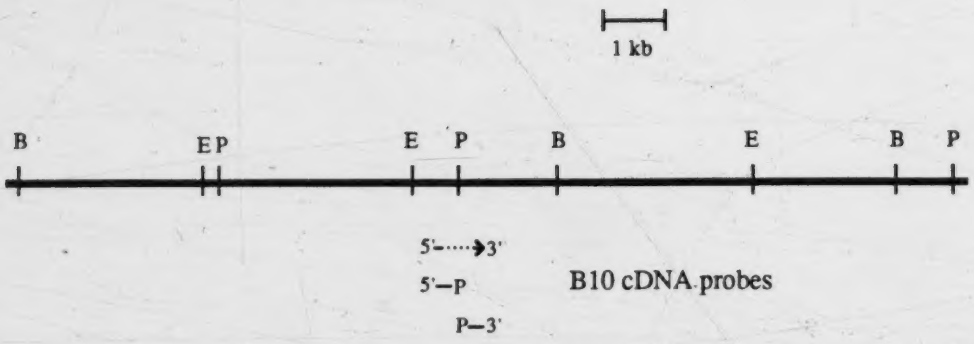


FIGURE IV.2 Southern blots of genomic DNA and the B10 and C11 genomic clones. (A) and (B) Genomic DNA was isolated from CBA/J mouse thymus cells, digested overnight with the indicated restriction enzyme, phenol/chloroform extracted and size fractionated on a 0.6% agarose gel. The genomic DNA was then transferred to nylon membranes and hybridized with (A) B10 cDNA insert and (B) the RsaI/Sau3A fragment from the C11 cDNA, corresponding to the 5' untranslated region. (C) and (D) DNA from the genomic clones, gB10 and gC11, was digested with various restriction enzymes, fractionated on 0.8% agarose gels, and transferred to nylon membranes. The Southern blot of gB10 was hybridized with the B10 cDNA insert, and the blot of gC11 with the C11 cDNA insert. Restriction enzymes used were EcoRI (E), BamHI (B), HindIII (H) and PstI (P).

A. B10 Gene



B. C11 Gene

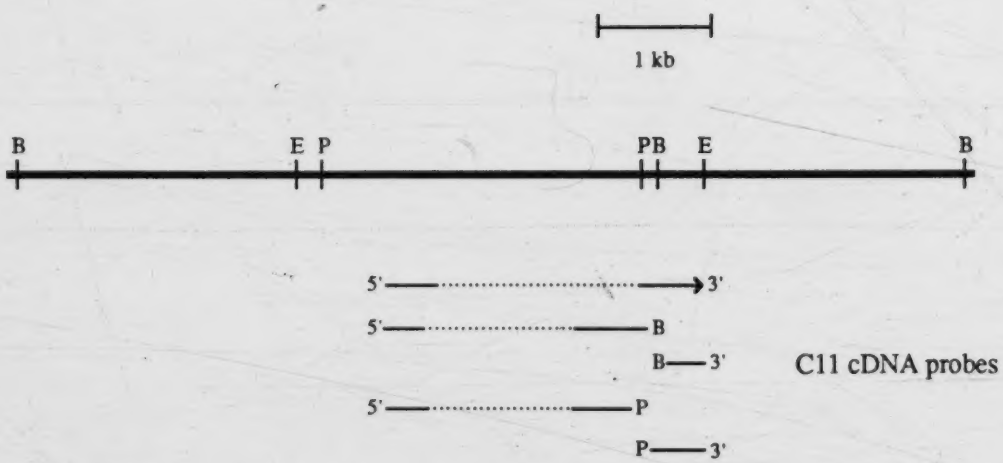


FIGURE IV.3 Restriction maps of the B10 and C11 genes. The cDNA probes used to map the genes are shown under each. The direction of transcription is also indicated. Note that the genes are drawn to two different scales. Restriction sites are BamHI (B), EcoRI (E) and PstI (P).

Although the 2 genes are homologous at the nucleotide level, their restriction enzyme patterns are not remarkably similar, except the BamHI/EcoRI/PstI sites in the 5' upstream region of the genes.

The orientation of the C11 cDNA clone relative to the restriction map of the gene was obtained by hybridizing each of the two BamHI and PstI cDNA fragments to the genomic Southern and comparing the bands hybridized by the 5' vs. the 3' fragments. Since the sequence of the cDNA, and consequently the strand which corresponds to the mRNA, is known, the direction of transcription of the gene could be deduced (figure 3B). To determine the direction of transcription of the B10 gene, the Southern blot of the cloned B10 gene (described below) was hybridized with each of the partially purified PstI fragments of the B10 cDNA insert (figure 4A and B). The PstI fragment which corresponds to the 5' end of the B10 transcript gave a signal for the 4.0 kb PstI band, the 0.7 kb EcoRI/PstI band and the 4.0 kb BamHI/PstI band. The PstI fragment which corresponds to the 3' end of the B10 transcript gave a stronger signal for the 8.1 kb PstI band, the 4.9 kb EcoRI/PstI band, and the 1.6 kb BamHI/PstI band. Therefore the orientation of the cDNA clone relative to the genomic map and the direction of transcription is as shown in figure 3A.

2. *Isolation of B10 and C11 genomic clones*

Genomic libraries were screened in order to obtain C11 and B10 genomic clones. Two million recombinants of a mouse genomic library in the lambda charon 4A vector (prepared by Drs. R.C. Bleackley and J. Bell) were screened in duplicate on 10 nitrocellulose filters, first by hybridization with radioactively labelled B10 insert. Only one duplicate positive signal was obtained. The corresponding recombinant phage is referred to as gB10. The filters were then washed and probed with radioactively-labelled C11 insert, which hybridized to the same recombinant as B10 did and also to one other clone. This

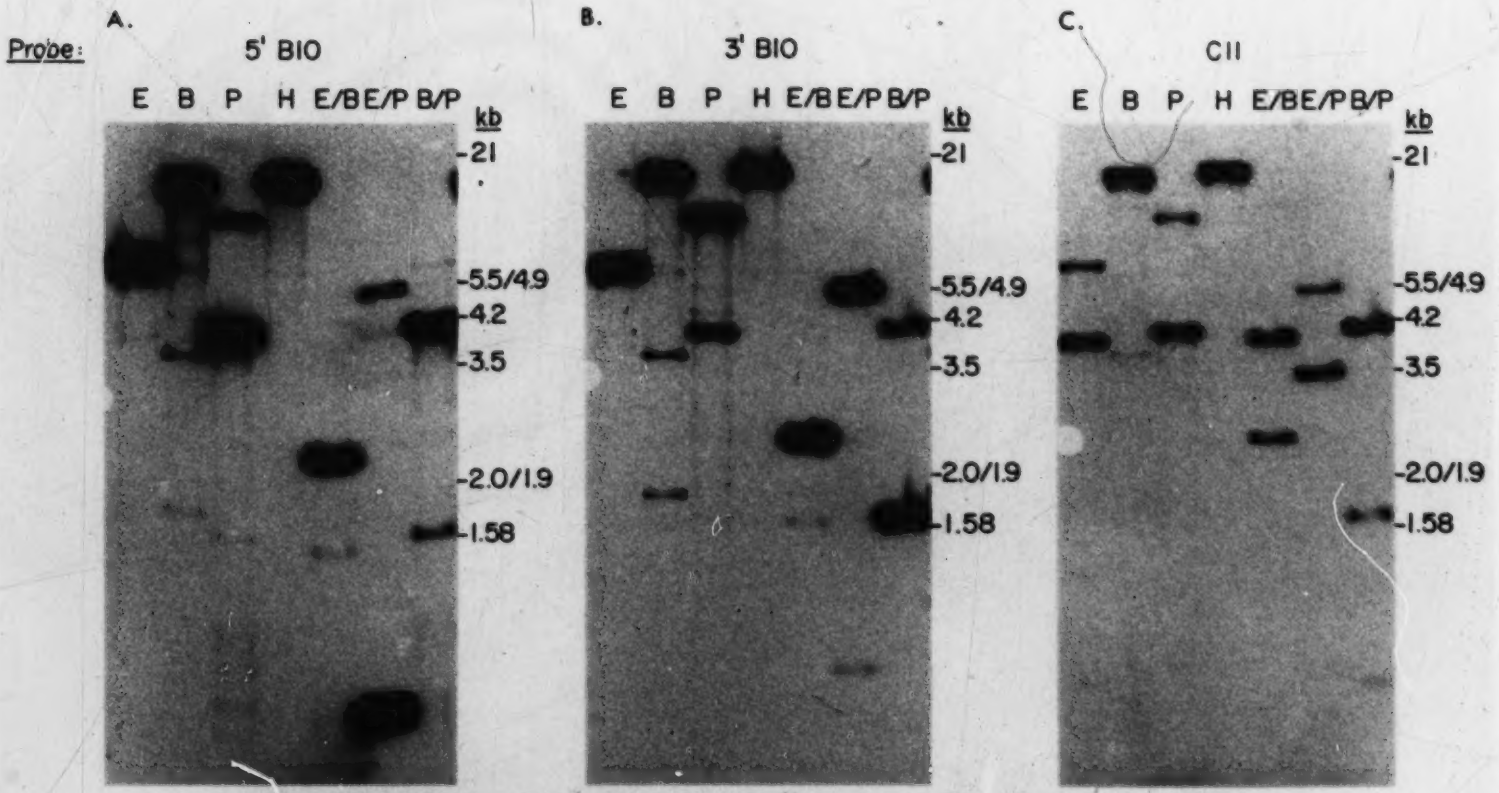


FIGURE IV.4 Direction of transcription of gB10 and homology to C11. The B10 genomic clone was digested with restriction enzymes as indicated, fractionated on a 0.8% agarose gel, and tranferred to nitrocellulose. The blot was then hybridized in turn with the nick-translated 5'- and 3'-PstI fragments of B10 insert cDNA and C11 insert cDNA.

second clone gave a weaker signal and restriction enzyme mapping revealed that it was neither genomic B10 nor C11, but another related sequence, designated gN1. This clone is described in the Chapter 3 appendix. Another murine genomic library was obtained from M.M. Davis (Stanford) and 2 x 10^6 recombinants from this library were screened in duplicate by hybridization with the C11 insert. Six positive signals appeared in duplicate, but upon re-plating and screening these six at lower density, only one hybridized with C11 in duplicate. This clone is referred to as gC11.

DNA was prepared from the gB10 and gC11 clones, the DNA was digested with restriction enzymes and Southern blots were prepared as for the genomic DNA. The Southern blot of the gB10 clone was hybridized with B10 cDNA insert, and the blot of gC11 was hybridized with C11 cDNA insert (figures 1C and D). The restriction pattern for each of the clones corresponds to the pattern seen when the cDNA clones were used to probe genomic DNA, which verified that these were indeed B10 and C11 genomic clones.

The initial restriction enzyme map for B10 was obtained by hybridizing Southern blots of genomic DNA with insert from the B10 cDNA probe. Because this insert only represents 380 bp at the 3' end of the B10 transcript, the map was incomplete at the 5' end of the gene. Therefore, the 9 kb BamH1, 5.6 kb EcoRI, and 4.0 kb and 8.1 kb PstI fragments from the gB10 clone were purified and used to probe the gB10 Southern blot. This approach is similar to genomic-walking, in that each restriction enzyme fragment hybridizes to overlapping fragments of the other restriction enzyme digests. This provided the additional information to extend the restriction map of the B10 gene. Finally, the same gB10 Southern blot was probed with C11 cDNA insert (figure 4C). The C11 probe hybridized both to the 3' fragments (5.6 kb EcoRI and 8.1 kb Pst I), and to the 5' fragments (3.6 kb EcoRI and 4.0 kb PstI) of the digested gB10. Therefore, the high degree of homology between the 3' regions of B10 and C11, which was demonstrated by sequence analysis (chapter III; Lobe *et al.* , 1986b), probably also extends to the 5' end of the genes.

3. *Exon/Intron organization of the C11 gene*

More detailed mapping of the C11 gene was afforded by subcloning the 3.6 kb EcoRI fragment of gC11 into pUC13. This permitted cleaner preparations and larger quantities of the fragment to be made than was possible with the entire genomic clone in the lambda vector. The 3.6 kb insert DNA from the subclone was digested with RsaI, RsaI+PstI and RsaI+BamHI and the fragments sized on a polyacrylamide gel. Two RsaI fragments, 907 bp and 500 bp, were cleaved by PstI and therefore corresponded to fragments at each end of the insert where the PstI sites occur (figure 5). Only one of these, the 907 bp band, was cut by BamHI, which implied that the 907 bp fragment is at the 3' end of the gene, and the 500 bp fragment is at the 5' end. The sizes of the sub-bands when these RsaI fragments were digested with PstI and BamHI are shown under the restriction enzyme map, and implied that they occur at the extreme ends of the 3.6 kb EcoRI fragment.[1] The same DNA digests were run on an agarose gel and a Southern blot was prepared. When the blot was probed with a C11 cDNA sequence which includes all but the 5' untranslated sequence (probe "d" in figure 5), the 907 bp as well as the 1650 and 255 bp RsaI fragments were hybridized. When probed with a C11 cDNA fragment, which extends from the start codon to an RsaI site upstream of the start codon (probe "b" in figure 5), only the 255 bp RsaI fragment was hybridized. Therefore, the 1650 bp fragment must be immediately upstream of the 907 bp fragment, and the 255 bp fragment is the next upstream of that. The blot was subsequently probed with a synthetic oligonucleotide (probe "a" in figure 5). This 20-mer is complementary to the sequence just upstream of the RsaI site in the untranslated 5' region. On the Southern blot, a low MW band hybridized to the 20-mer which aligned with the 195 bp RsaI fragment. However, on an agarose gel the

[1]An alternative order for the 5' end fragments is a 195 bp EcoRI to RsaI fragment, 150 bp RsaI to PstI, 350 bp PstI to RsaI, followed by 50, 55 and 90 bp RsaI fragments. In this case, the 350 bp band in the EcoRI/PstI digest would be replaced by a different 350 bp band in the EcoRI/PstI/RsaI digest.

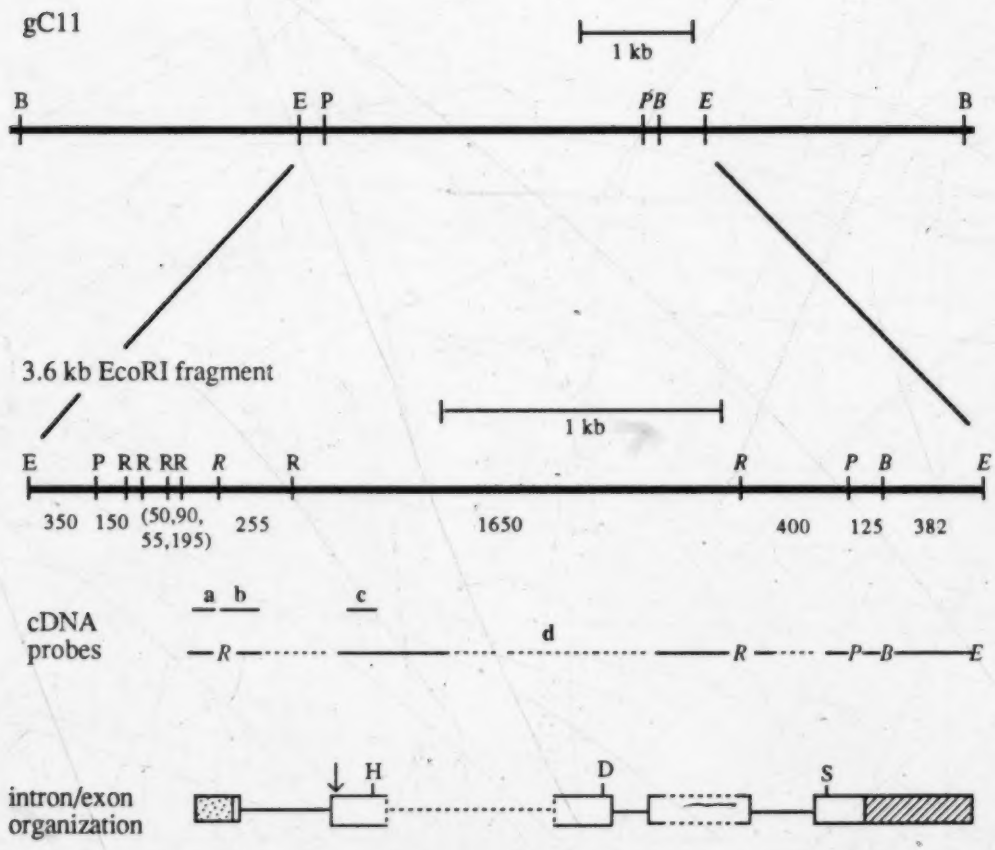


FIGURE IV.5 Organization of the C11 gene. The 3.6 kb EcoRI fragment of gC11was isolated, digested with RsaI (R), PstI (P) and BamHI (B), and the fragments were sized on a 5% polyacrylamide gel. The digests were also run on a 1% agarose gel, transferred to nitrocellulose and hybridized with a synthetic 20-mer (a) and cDNA fragments (b, c and d) as described in the text. The order of the RsaI fragments, based on the Southern blot hybridizations, is shown under the map of the EcoRI fragment (numbers indicate size in bp). The restriction enzyme sites which correspond to sites in the cDNA clone are printed in italics. The preliminary map of the exon/intron organization of the C11 gene is shown at the bottom of the figure, with exons drawn as open boxes and introns as lines. The DNA sequence encoding the signal peptide is denoted by a dotted bar and the 3' untranslated region of the gene by a slashed bar. The codon for the residue at the site where the activation peptide is cleaved is indicated by a vertical arrow. H (His-44), D (Asp-88) and S (Ser-183) indicate the positions of the codons for the catalytic triad residues. Regions in which the exon/intron organization is not known are drawn in dotted lines.

small fragments were not resolved well enough to be certain of which of the small bands is indicated in brackets, since their order remains uncertain.

From a comparison of the location of the RsaI sites and the size of the fragments in the genomic clone to the sites in the cDNA clone, the regions in which introns occur in the gene could be deduced (figure 5). Since there is only one RsaI site in the coding region of the gene, it must correspond to the single RsaI site in the translated sequence of the cDNA. One other RsaI site occurs in the cDNA, in the 5' untranslated sequence, 100 bp upstream of the start codon. The corresponding site in the genomic clone is at the 5' boundary of the 255 bp RsaI fragment. The RsaI site in the gene which is at the 3' boundary of this 255 bp fragment does not have a matching site in the cDNA clone so it must occur in an intron. Therefore, an intron begins within this 255 bp RsaI fragment. At least one intron must also exist in the region of the codons for His^{44} and Asp^{88}, two members of the catalytic triad (Lobe *et al.*, 1986b), since that RsaI fragment is 1650 bp in the genomic clone, but only 600 base pairs in the cDNA clone. Another intron must occur between the RsaI site and the PstI site in the coding region of the gene, again because the genomic fragment is larger than the corresponding cDNA fragment. This region encompasses the Ser^{183} codon, the other member of the catalytic triad. These RsaI fragments of the gC11 clone were subcloned into the M13 vector for sequence analysis. Preliminary data indicates that one intron occurs at ntd 70 in the cDNA sequence, shown in figure III.1, which splits the sequence thought to encode the activation peptide. Another intron occurs at ntd 352, just downstream from the Asp^{88} codon. This intron has been sequenced and is 132 bp long. Thus, at least one other intron must occur in this RsaI fragment to account for the additional 1000 bp of non-coding sequence, probably between the His^{44} and Asp^{88} codons. The intron which was implicated to occur in the 400 bp RsaI/PstI sequence, in the vicinity of the Ser^{183} codon was found by sequence analysis to occur at ntd 613.

The pattern of exon/intron arrangement for the C11 gene was compared to that of other serine protease genes (figure 6). The serine protease genes and the homologous

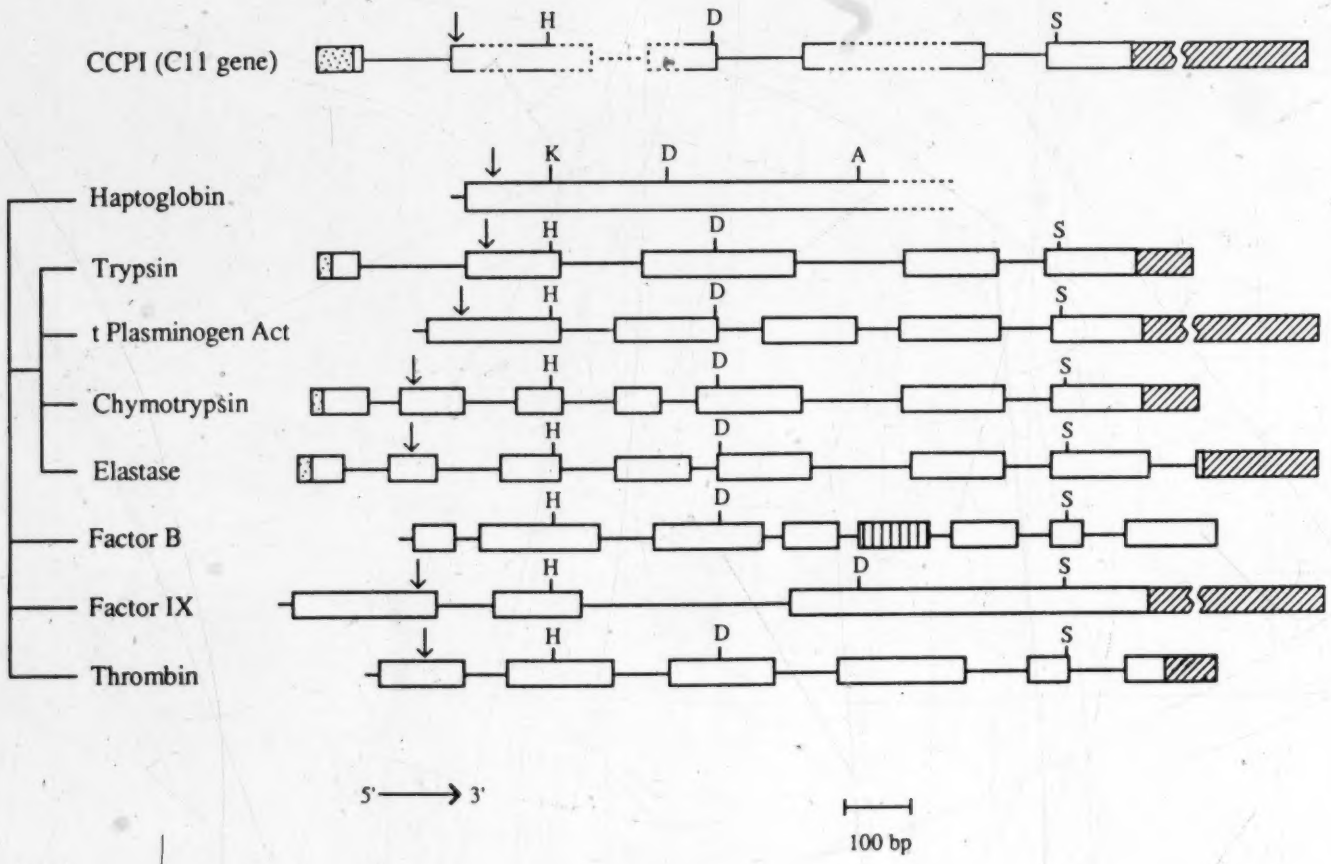


FIGURE IV.6 Comparison of the C11 gene to other serine protease genes. The CCP-encoding C11 gene is shown at the top, and below it are serine protese genes representing each of the 5 groups, distinguished by gene organization (Rogers, 1985). Locations of the codons for the active site residues (His, H, Asp, D, and Ser, S) are shown. The vertical arrow designates the point of cleavage of the activation peptide. Complement factor B is not activated in this way. The signal peptide is denoted as a dotted bar, the 3' untranslated region as a slashed bar, and the unique exon of complement factor B as a vertically-striped bar. Exons are drawn to the scale shown, but introns are not drawn to scale.

haptoglobin gene can be grouped into 5 different types, based on the intron position in the catalytic region of the genes. This grouping is thought to reflect the evolution of the genes by exon shuffling and intron insertion (Rogers, 1985), and is also consistent with the amino acid sequence homologies between the serine proteases (Irwin and MacGillivray, 1987). The C11 gene organization determined so far shows that it possesses the features of the trypsin gene family, which includes the pancreatic serine proteases, kallikrein, the alpha and gamma subunits of the nerve growth factor, tissue-type plasminogen activator, and urokinase.

4. *B10 and C11 mRNAs are regulated at the level of transcription*

The regulation of B10 and C11 mRNA levels could occur at the level of transcription of the genes or by alteration of the half-life of the mRNAs. Nuclear run-off transcription experiments have shown that at least part of the increase in B10 and C11 mRNA in activated CTL is due to an increase in transcription (Jenny Shaw, unpublished). Nuclei were isolated from spleen cells at days 0, 1, 2 and 3 after ConA stimulation. *In vitro* transcription was allowed to proceed in the presence of ^{32}P-UTP under conditions in which no new initiation of transcription would occur (Kronke *et al.*, 1984). Therefore, only RNA synthesis which was in progress at the time the nuclei were isolated was labelled. The labelled RNA was then hybridized to C11, B10 and actin cDNA, immobilized on nitrocellulose filters. By autoradiography of the filters, the level of transcription of the B10 and C11 genes at a given point in time, represented by the amount of RNA bound to the respective cDNA clone, could be visualized. This revealed that the level of ongoing C11 transcription increased and peaked at 2 days post-ConA stimulation, and of B10 at 2 to 3 days post-ConA stimulation. Therefore, all or part of the increase in the levels of C11 and B10 mRNA upon activation of CTL can be attributed to an increase in transcription of the genes.

5. DNase I Sensitivity of the B10 and C11 genes

Genes which are being actively transcribed have been associated with an altered chromatin configuration, which renders the DNA more susceptible to pancreatic DNase I digestion (Weisbrod, 1982). Furthermore, regions of the gene which are involved in transcriptional regulation have been found to coincide with DNase I "hypersensitive" sites, which probably reflects the accessibility to regulatory proteins (Elgin, 1982). Since regulation of B10 and C11 expression was found to occur via an increase in transcriptional activity, the DNase I sensitivity of the genes was examined.

Nuclei were isolated from different cell types and treated with increasing concentrations of DNase I for 15'. The DNA was then purified by proteinase K digestion and phenol/chloroform extractions, followed by digestion with BamH1. This restriction enzyme was used because it divides the C11 gene into a 5.7 kb 5'-fragment, extending 2.8 kb upstream of the gene, and a 2.7 kb 3'-fragment, thus allowing a comparison of the 5' and 3' regions of the gene. In the case of B10, the entire gene probably resides on the 9 kb Bam H1 fragment, as judged by cross-hybridization with the full-length C11 cDNA. Therefore the B10 cDNA, which only includes the 3' part of the gene, could be used as a probe for the BamHI-digested B10 gene.

Following restriction enzyme digestion, the DNA was fractionated by agarose gel electrophoresis and transferred to nitrocellulose. The Southern blots were then probed with radioactively-labelled full length C11 cDNA insert, or its BamHI fragments to detect the 5' or 3' half of the gene, and B10 cDNA insert. Some of the blots were also probed with a globin DNA sequence, which represents a gene not being transcribed in the cells tested.

The autoradiographs of the Southern blots are shown, together with the data from densitometric scanning of the autoradiographs. Although the densitometric scanning of the results provides a means to quantitate the data, it should be noted that the autoradiographs with faint signals tend to give imprecise readings. In analyzing the results, a consideration of both forms of the data presentation should be taken.

(a) *MTL 2.8.2 and 2.2.1 cells*

The cell line MTL2.8.2 expresses high levels of both C11 and B10 mRNA (Lobe *et al.*, 1986a). When nuclei isolated from these cells were DNase-treated, some very interesting differences were seen in the susceptibility of the 5' C11, 3' C11, B10 and globin DNA to DNase I (figure 7). The Southern blot was first probed with the full length C11 cDNA insert, which hybridized to both the 5' and 3' BamHI fragments. The 5' C11 band begins to decrease in intensity at 0.5 ug/ml DNase I and has disappeared at 8 ug/ml. The 3' C11 band, however, has not begun to decrease in intensity at 0.5 ug/ml, and is still visible at 8 ug/ml DNase I. The blot was washed and re-probed with B10 cDNA. The B10 BamHI band showed a similar rate of degradation to the 5' C11 band. It begins to decrease in intensity at 1.0 ug/ml DNase I and has almost disappeared by 8 ug/ml. The blot was next hybridized with a globin gene sequence. The bands corresponding to the globin gene showed very little digestion by DNase I , even at 8 ug/ml. These results are illustrated in the graph at the bottom of figure 7. The BamHI 5' C11 and B10 fragments, which demonstrate the greatest sensitivity to DNase I , both presumably contain the promoter regions. The BamHI 3' C11 fragment corresponds to DNA which is transcribed but probably does not contain promoter elements. This band is less sensitive to DNase I than the 5' C11 fragment. Lastly, the signal corresponding to the globin gene, which is not being transcribed, shows the least susceptibility to DNase I .

In addition to the differences in the overall sensitivity of the fragments to digestion, the 5' C11 and B10 probes hybridized to sub-bands, which indicates the presence of DNase I hypersensitive sites in the gene. This is clearly demonstrated for the 5' region of the C11 gene in figure 8A. When the main band begins to disappear at 0.5 ug/ml DNase, two bands begin to appear, at 2.9 and 2.5 kb. The 2.9 kb band only persists up to 1.0 to 1.5 ug/ml DNase I. The smaller band continues to increase in intensity as the main band disappears. These sub-bands represent specific hypersensitive DNase I cleavage sites. As

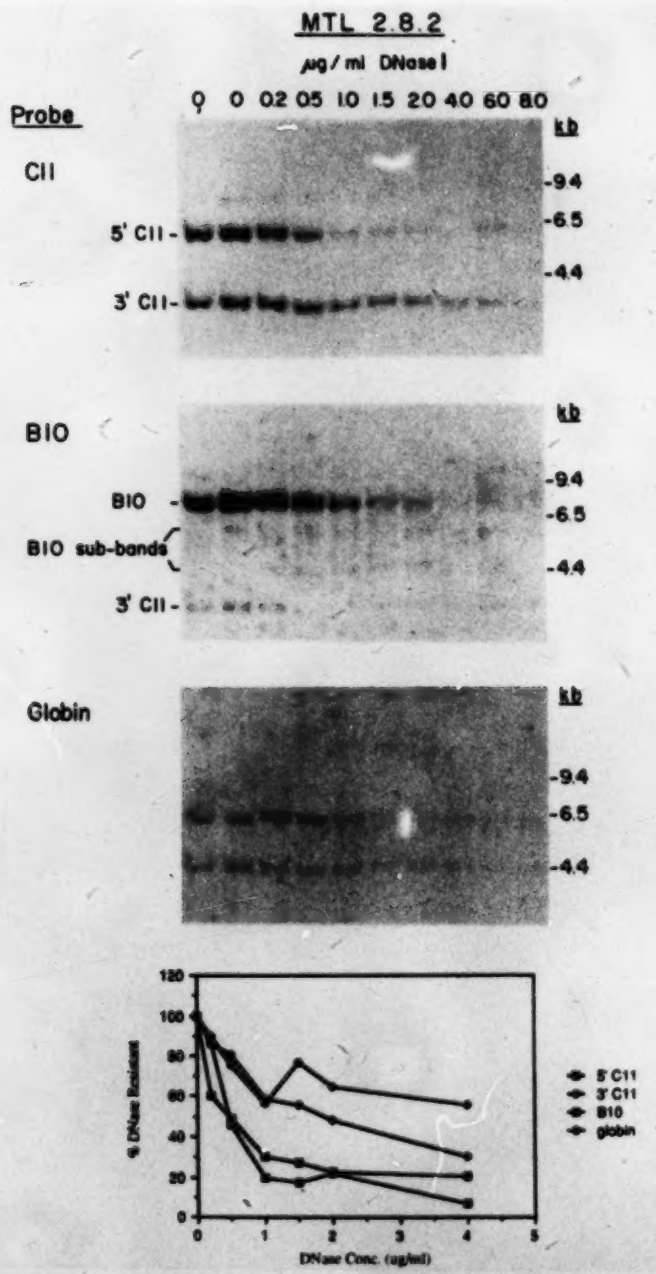


FIGURE IV.7 DNase I sensitivity of the C11 and B10 genes in MTL2.8.2 cells. Nuclei were prepared from a CTL line, MTL2.8.2, and incubated with increasing amounts of DNase I (indicated at the top of each lane). The first lane represents an unincubated sample, and the second lane is an incubated sample with no DNase I added. The DNA was isolated, digested with BamHI, fractionated on a 0.8% agarose gel and transferred to nitrocellulose. The Southern blot was hybridized with nick-translated C11 cDNA insert, washed and reprobed with the B10 cDNA insert, and finally, washed again and reprobed with a globin gene probe. The bands corresponding to the C11 gene BamHI fragments, the B10 gene BamHI fragment and the B10 sub-bands are indicated. The lanes of the autoradiographs were scanned on a densitometer to quantitate the intensity of the bands. The values for each of the bands, as a percentage of the 0 ug/ml DNase I value, were plotted against the concentration of DNase I, shown in the graph at the bottom.

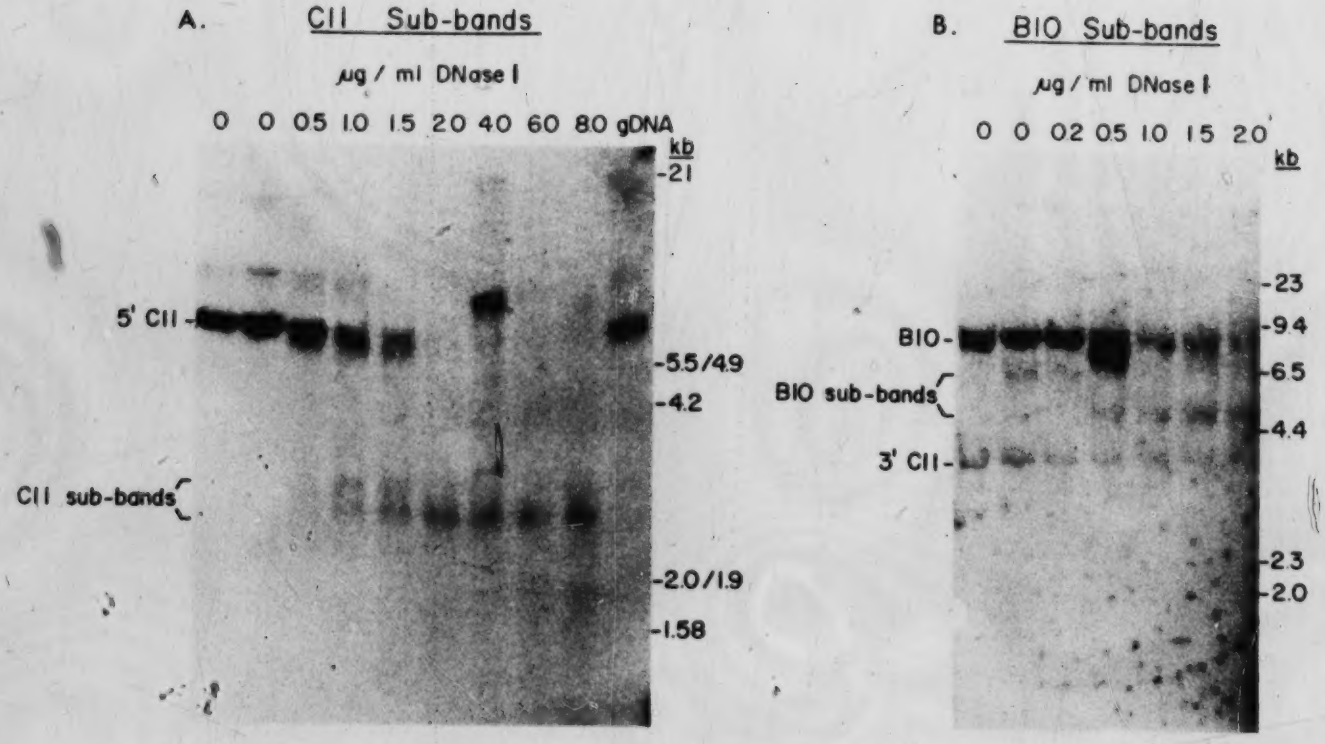


FIGURE IV.8 Appearance of C11 and B10 sub-bands due to DNase I hypersensitive sites. Nuclei were isolated, DNase I treated and BamHI digested, as described in figure IV.7. (A) C11 sub-bands in DNA from the CTL line MTL2.8.2. The southern blot was probed with the nick-translated 5' BamHI fragment of the C11 cDNA insert. The 5.7 kb band corresponding to the BamHI 5' C11 gene fragment and the two C11 gene sub-bands are indicated. 'gDNA' is BamHI digested genomic thymus DNA, not treated with DNase I. (B) B10 sub-bands in DNA from the CTL line, MTL2.2.1. The southern blot was hybridized with the nick-translated B10 cDNA insert. The positions of the 9 kb BamHI fragment of the B10 gene, and the B10 sub-bands are marked. In addition, the 3' BamHI fragment of the C11 gene is seen, due to cross-hybridization. The high MW band in the 4 ug/ml DNase lane of panel A and in the 0.5 ug/ml DNase lane of panel B is probably contamination with phage DNA.

these sites are preferentially attacked by DNase I , the main 5.7 kb BamHI band detected by the C11 cDNA is shortened to the 2.9 and 2.5 kb bands. The hypersensitive sites are therefore located 2.9 and 2.5 kb upstream of the BamHI site in the gene. This corresponds to the 5' upstream region of the C11 gene (figure 9A).

The B10 sub-bands are seen in figure 7, middle panel (MTL2.8.2 cells) and in figure 8B (MTL2.2.1 cells, discussed below). Again, as the main B10 band disappears, two sub-bands appear. The first sub-band appears at 0 ug/ml DNase I (digestion is due to endogenous DNase) and is 6.4 kb in size. At 0.2 ug/ml DNase I a second sub-band appears which is 4.5 kb in size. These sub-bands correspond to fragments which extend upstream from the 3' BamHI site (figure 9B; for both C11 and B10, the sub-bands must extend from the BamHI sites at the 3' end of the gene, or they would not overlap and be detected by the cDNA probes). Since the location of the 5' end of the B10 gene is not yet known, the relation of the hypersensitive sites to the promoter region is uncertain. However, they are located in positions very likely to be upstream regulatory sequences.

Another CTL line which expresses B10 and C11, MTL2.2.1, was assayed. The same DNase I-sensitivity of the C11 and B10 genes was observed, and the sub-bands due to the hypersensitive sites also appeared. An autoradiograph of a Southern blot of DNase I -treated MTL2.2.1 DNA probed with B10 is shown in figure 8B. The main BamHI fragment and sub-bands are indicated.

It appears, then, that the C11 and B10 genes, in cells which express them, have an overall DNase I-sensitivity relative to the non-expressed globin gene. In addition, DNase I hypersensitive sites occur in the 5'-upstream region of the genes which probably represent regulatory sequences.

(b) *Thymus cells*

The chromatin conformation in the region of the C11 and B10 genes was also examined, using DNase I , in thymus cells. The thymus is the site of maturation of T cells

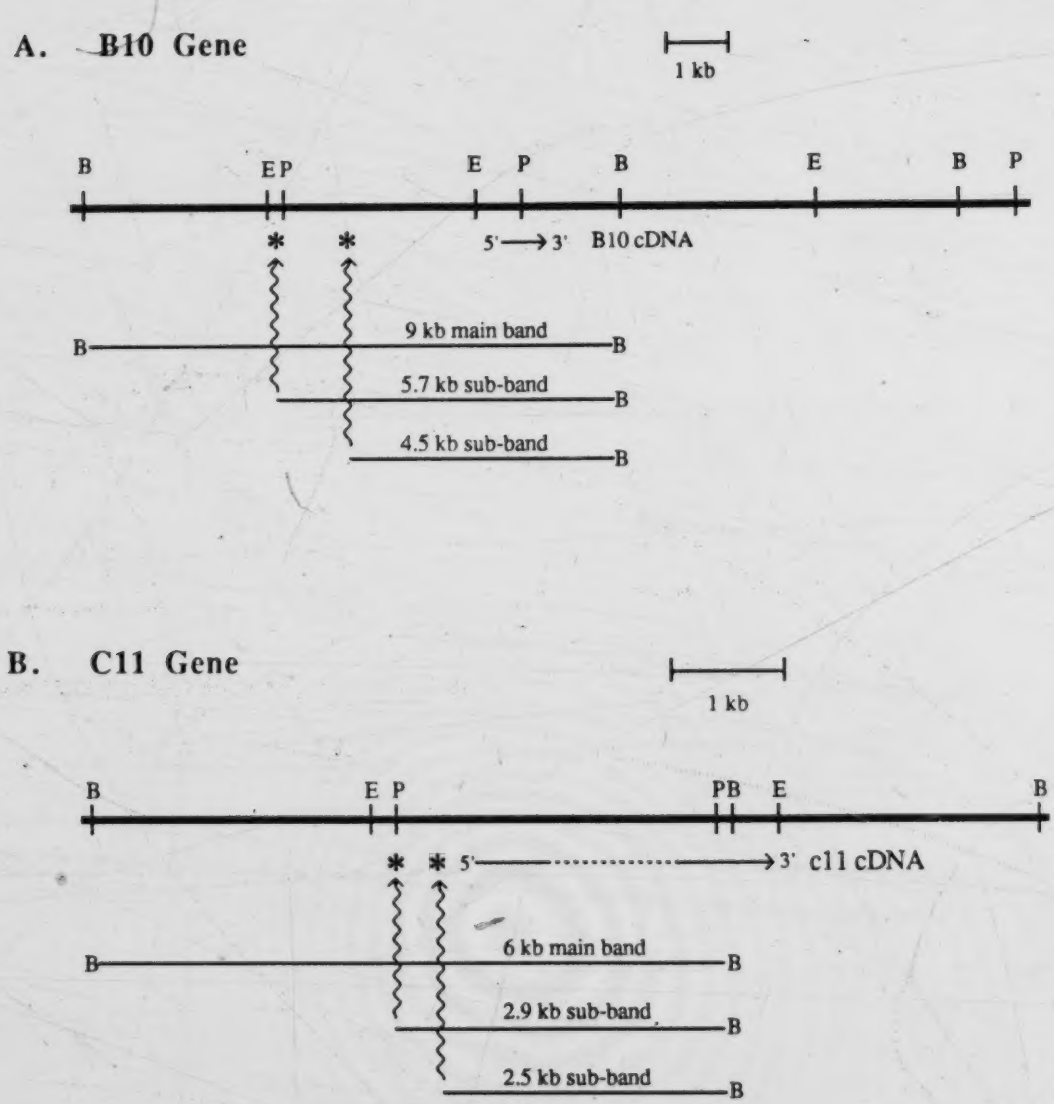


FIGURE IV.9 Location of the DNase I hypersensitive sites in the B10 and C11 genes. The restriction map of each gene is shown with the direction of transcription and the cDNA probes used underneath. Below that, the BamHI fragment is shown for each gene, as well as the shorter sub-fragments. The location of the DNase I hypersensitive site corresponding to each sub-fragment is denoted with an asterisk.

and contains a mixture of immature T-cells, as well as epithelial cells. Thymus cells were obtained from 11 week-old CBA/J mice and used for a nuclei preparation, as described for the MTL2.8.2 cells. Thymocyte nuclei were found to be quite fragile relative to the other cells. For some experiments (not shown), this problem was overcome by replacing NP40 with saponin in the lysis buffer, as it is a milder detergent.

The Southern blot was probed with the C11 cDNA insert, which hybridized to both the 5' and 3' BamHI bands of C11, and also cross-hybridized with the B10 gene fragment (figure 10). All three bands only begin to decrease in intensity at 4 ug/ml DNase I, and are still visible at 8 ug/ml DNase I. The blot was washed and probed with globin, which showed the same rate of degradation as the other genes. The DNase I concentrations at which disappearance of all the bands occur in thymus is similar to the 3' C11 and globin genes in the MTL lines, so the C11 and B10 genes do not appear to be DNase I sensitive in thymus cells.

Although on a Northern blot the RNA from these cells was found not to contain the C11 or B10 transcripts (data not shown), the populaton of thymocytes consists primarily of cells committed to the T cell lineage, which might be expected to have the potential to express the B10 and C11 genes. This potential is usually realized as an unfolding of chromatin structure, making the genes available for transcription factors, as revealed by an increased DNase I sensitivity. The B10 and C11 genes from thymus cells, however, do not exhibit this DNase I sensitivity. Possibly, the gene does not become available for transcription until the cells have further differentiated and become committed to the killer T cell (T_K) subset. The thymocytes are a mixture of T cell subsets and are not fully differentiated cells. Therefore those which are of the pCTL subset and are at the stage of differentiation where they are capable of expressing the C11 and B10 genes, may not represent a large enough fraction of the population to cause a noticeable increase in DNase I susceptibility of the C11 and B10 genes.

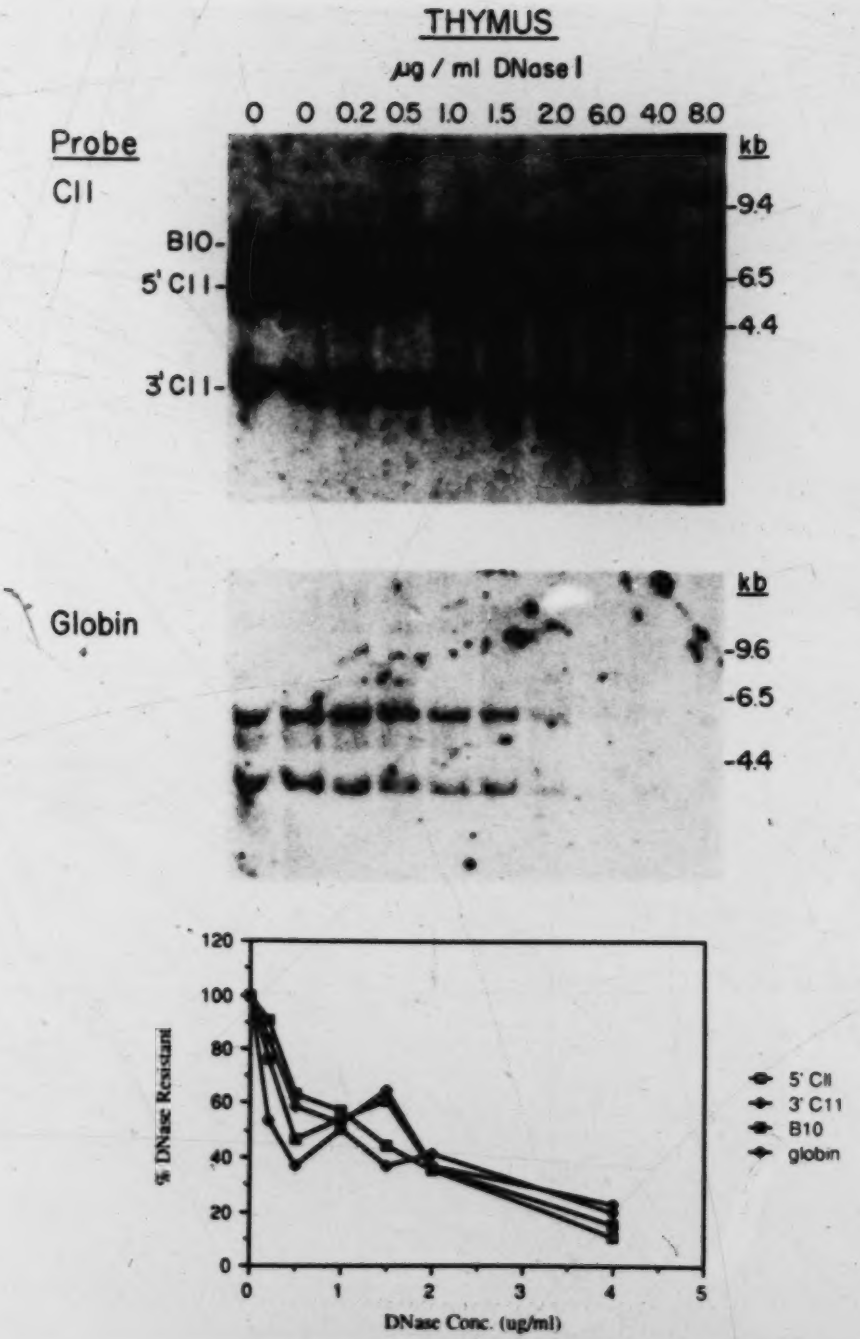


FIGURE IV.10 DNase I sensitivity of the B10 and C11 genes in thymus cells. Nuclei were isolated from thymus cells, DNase I treated and BamHI digested, as described in figure IV.7. The Southern blot was probed with nick-translated C11 cDNA insert, which hybridized with both the C11 and B10 gene fragments, as labelled. The blot was subsequently washed and re-hybridized with a globin gene probe. The values of the band intensities were obtained by scanning densitometry and graphed as for figure IV.7.

(c) *CTL-L16 cells*

Another type of cell assayed was the T cell line, CTL-L16. These cells were once cytotoxic, but since being cloned have lost their cytotoxic activity. They still contain the characteristic granules of cytotoxic cells, however when RNA from these cells was tested for the presence of C11 and B10 transcripts, the level was found to be ~50-fold lower than in MTL2.8.2 cells. The C11 protein product, CCPI, was also not detected in CTL-L16 cells on a Western blot (Redmond *et al.*, submitted). Thus, these cells seem to have changed, while being cultured, from functional CTL which probably expressed B10 and C11, to non-functional cells which no longer produce some molecule(s) critical to their lytic activity. We were interested to see if these cells, which no longer express the C11 and B10 genes, possess the DNase I sensitivity and hypersensitive sites seen in the other CTL lines which do express C11 and B10. Although the CTL-L16 cells are also not cytotoxic in the presence of the mitogen, ConA, T cells generally do become activated by ConA. Therefore half of the cells were treated with ConA for 16 hrs prior to nuclei isloation, to determine if the mitogen would induce any conformational changes in the chromatin.

Nuclei were DNase I-treated, the DNA cleaved with BamHI and a Southern blot of the DNA was prepared, as for the previous cell types. The blot was first probed with the C11 cDNA (upper panel, figure 11). The 5' BamHI fragment of the C11 gene has disappeared by incubation with 1 ug/ml DNase, whereas the 3' fragment is still visible at 2 ug/ml. The result is the same for the ConA-treated cells. The graph of the densitometric scan of the ConA-treated cells, shown at the bottom of the figure, illustrates the slightly faster degradation of the 5' fragment than the 3' fragment. Overall, the C11 gene appears to be more susceptible in the CTL-L16 cells than in the MTL2.8.2 cells. Even the 3' end of the gene is already at a low level at 1 ug/ml DNase I for CTL-L16, but not until 6 to 8 ug/ml for MTL2.8.2 cells. This is not simply due to a greater permeability of CTL-L16 cells to DNase I , nor to a higher content of endogenous nucleases, since the B10 gene is no more sensitive than in MTL2.8.2 cells. However, the C11 sub-bands do not appear as

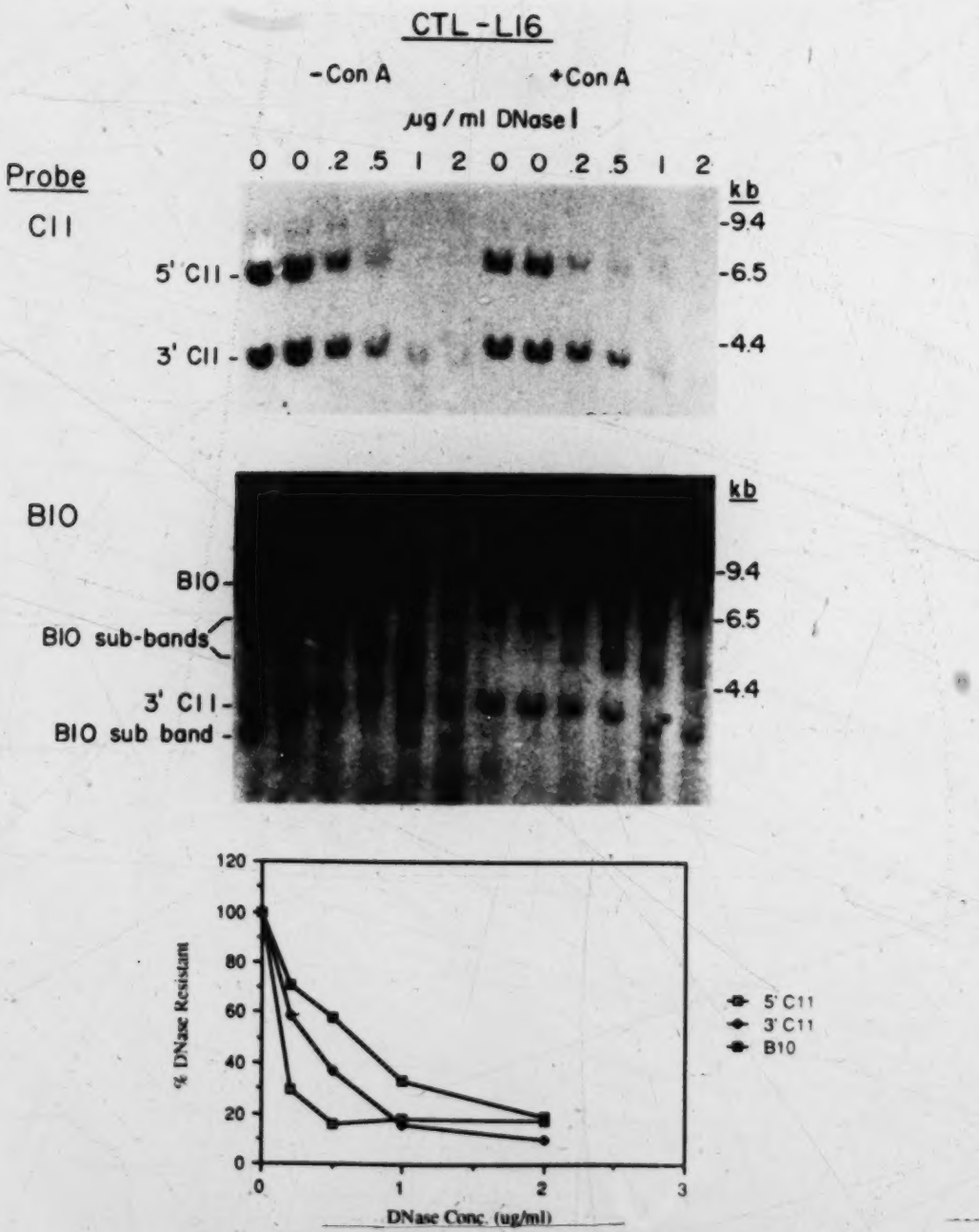


FIGURE IV.11 DNase I sensitivity and hypersensitive sites in a non-cytolytic CTL line, CTL.L16. Nuclei were isolated from CTL.L16 cells, with or without a prior 16 hr ConA incubation. After DNase I treatment and Bam HI digestion, as described in figure IV.7, the DNA was size-fractionated on a 0.8% agarose gel, and transferred to nitrocellulose. The Southern blot was hybridized with nick-translated C11 cDNA insert and with B10 cDNA insert. The BamHI bands corresponding to the 5' and 3' C11 and the B10 fragment are labelled, as well as the B10 sub-bands. The band intensities, as determined by scanning densitometry, were again plotted against the DNase I concentration, shown in the graph at the bottom.

the DNase I concentration is increased. Perhaps because of the greater overall sensitivity of the gene, the random DNase I nicks occur too quickly to allow visualization of the sub-bands which are due to the hypersensitive sites. A greater number of DNase I concentrations between 0 and 1 ug/ml DNase I needs to be tested to see if there is a window in which the sub-bands do appear.

When the blot was washed and re-probed with the B10 cDNA, a more complex pattern of hybridization appeared (figure 11, middle panel). Although it was not as sensitive as the C11 5' gene fragment, as seen on the graph, the B10 BamHI gene fragment was equally susceptible to DNase I in CTL-L16 cells as in MTL2.8.2 cells. Three different sub-bands appear on the autoradiograph, both for ConA-treated and untreated cells. The largest two are the same as the sub-bands seen in MTL2.8.2 and MTL2.2.1 cells, which represent the two hypersensitive sites indicated in figure 9B. As for the MTL lines, the 6.4 kb band begins to appear when the nuclei are incubated with no exogenous DNase I (0 ug/ml DNase I), so the site is susceptible enough for endogenous nucleases to selectively attack it. In the two lanes representing 0 ug/ml DNase I, the faint 6.4 kb band is difficult to distinguish as the B10 probe is cross-hybridizing to the 5' C11 BamHI fragment at 5.7 kb. However, as seen with the C11 probe, the 5' C11 band has faded at 0.2 ug/ml DNase I, whereas the B10 sub-band, which is slightly above the position for the 5' C11 band, persists. Digestion at the hypersensitive site further downstream begins at 0.2 ug/ml DNase, and the 4.5 kb sub-band intensity peaks at 0.5 to 1.0 ug/ml. This leads to a decrease in the amount of the 5.7 kb fragment due to conversion to the 4.5 kb fragment. At 1.0 ug/ml DNase, a third band appears which may be another B10 sub-band. It appears in the DNA from cells with or without ConA addition, although it is more apparent in the untreated cells. This sub-band is ~2.3 kb, which would place the corresponding hypersensitive site just upstream of the sequence complementary to the B10 cDNA, and consequently within the B10 gene. In the process of cloning, subcloning and sequencing the B10 and C11 sequences, this region was found to be prone to unusual recombinational

events, probably due to unusual secondary structure. The region causing these difficulties lies in the middle of the transcript just upstream of the Ser^{183} codon. Whether the same sequence is rendering the gene DNase I hypersensitive and whether this serves a physiological function, such as allowing a regulatory factor to bind, remains to be seen. One suspicion regarding this sub-band that should also be noted is that the 2.3 kb signal may be a contamination with the 2.3 kb plasmid, pUC13, especially considering the strong signal seen in the first lane (-ConA, 0 ug/ml DNase).

Whether the 2.3 kb sub-band is real or not, the higher MW bands demonstrate that the B10 gene is DNase-sensitive in CTL-L16 cells, and that the same B10 hypersensitive sites are present as in the MTL lines. The C11 gene is also hypersensitive to DNase I. The CTL-L16 cells, then, seem to have the potential to transcribe the genes, but have lost a signalling molecule required to induce expression. The lack of appearance of the C11 sub-band may be due to the very high overall-DNase I sensitivity of the region, or it may be related to the lack of binding of a transcripton factor absent in CTL-L16 cells.

(d) *EL4 cells*

The DNase I-sensitivity of the B10 and C11 genes was next examined in a non-cytolytic T cell line, EL4 (Farrar *et al.*, 1980). This thymoma line produces lymphokines when stimulated with phorbol myristate acetate (PMA) and so resembles a helper cell. The B10 and C11 genes are not transcribed in these cells (chapter II; Lobe *et al.*, 1986a). Nuclei were isolated from EL4 cells and DNase I-treated, as for the other types of cells. The DNA was BamHI-digested and used for a Southern blot. The blot was then successively hybridized with the 5' C11 BamHI fragment, the 3' C11 Bam HI fragment and, finally, with B10 (figure 12). Surprisingly, in this cell line the 5' C11 and B10 BamHI bands are more susceptible to DNase I than the 3' C11 band. Whereas the 5' C11 and B10 signals fall at 0.5 ug/ml, the 3' C11 does not decrease until 2.0 to 4.0 ug/ml DNase I. This is different from the situation in thymus in which all of the bands were

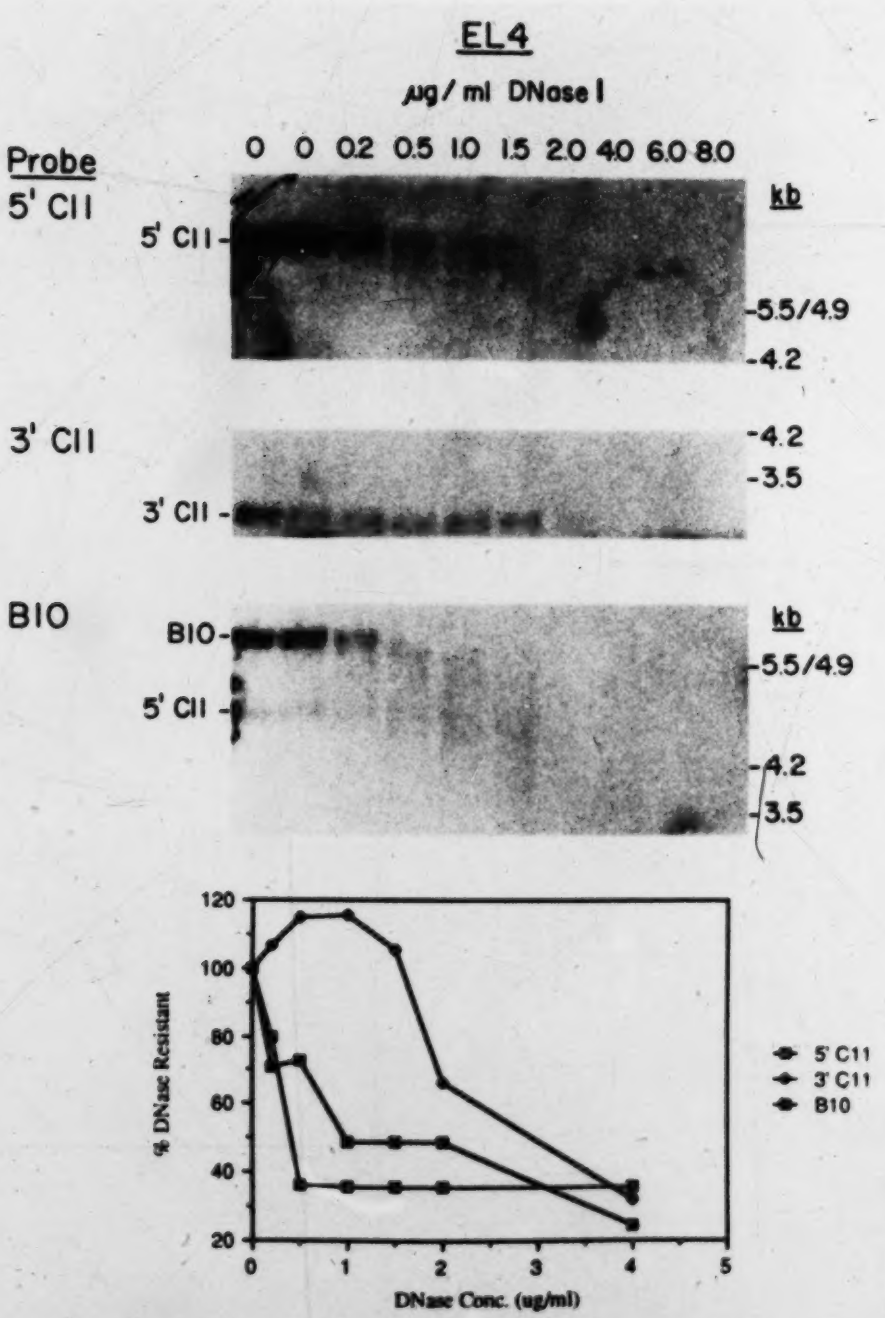


FIGURE IV.12 DNase I sensitivity of the C11 and B10 genes in a T cell thymoma, EL4. Nuclei were prepared and DNase I-treated as described in figure IV.7. After Bam HI digestion, Southern blots of the DNA samples were prepared, and hybridized sequentially with the 5' and 3' BamHI fragments of C11 cDNA and the B10 cDNA insert, radiolabelled by nick-translation. The BamHI fragments of the C11 and the B10 genes are labelled. The band intensities were determined by densitometric scanning and plotted as a function of DNase I concentration, as in figure IV.6 (bottom panel).

degraded at the same rate, beginning at 4.0 ug/ml DNase I. Although this suggests the genes may have the promoter-related hypersensitive sites seen in the MTL and CTL-L16 lines, the corresponding sub-bands do not appear. In fact, the DNase I seems to be nicking the 5' region of C11 and B10 randomly, since the bands become diffuse at 0.5 to 1.5 ug/ml DNase I before disappearing at 2.0 ug/ml. Thus, it seems that in this cell line, which is a differentiated T cell but of the helper (T_H) rather than the killer (T_K) cell subset, the 5' regions of the C11 and B10 genes have an overall DNase I sensitivity, but do not have the hypersensitive sites seen in the T_K cells. It may be that in all differentiated T cells, the 5' region of the B10 and C11 genes are accessible, and the difference in expression occurs via the presence or absence of a CTL-specific protein which activates transcription.

(e) *S194 cells*

A myeloma cell line, S194, was analyzed in the same way in order to compare the DNase I susceptibility of the B10 and C11 genes in T cells to the situation in a B cell line (figure 13). In this cell line, the 5' C11, 3' C11 and B10 gene fragments remain at a constant level until the DNase I is at 4 to 6 ug/ml. At this concentration, all three bands become diffuse, and have disappeared at 8 ug/ml. This is similar to the rate of degradation in the heterogeneous thymus cell population, where the genes were no more DNase-sensitive than the globin gene. The graph of the data is somewhat misleading, since the 0 ug/ml DNase I samples had more DNA and the drop in intensity from 0 to 0.2 ug/ml DNase I is exagerated. The persistance of all three bands up to 4 to 6 ug/ml of DNase I and the equal sensitivity of the 5' and 3' C11 Bam H1 regions imply that the genes are not DNase I sensitive in a B cell line.

D. DISCUSSION

A restriction map for the C11 and B10 genes was constructed by hybridizing Southerns of genomic DNA with cDNA probes. In spite of the extensive sequence

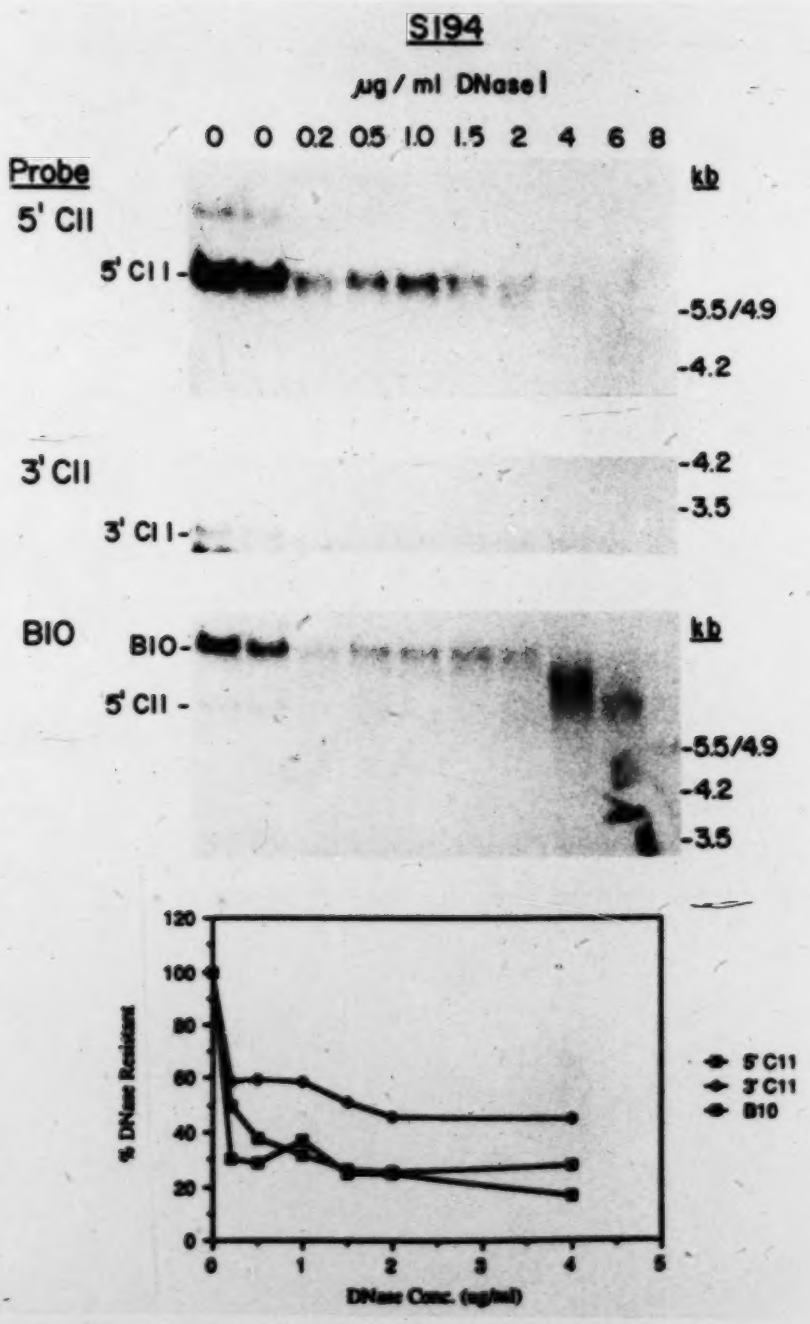


FIGURE IV.13 DNase I sensitivity of the C11 and B10 genes in a B cell myeloma, S194. Nuclei were isolated and DNase I treated, as for the other cell types. After BamHI digestion, a Southern blot was prepared, which was hybridized first with the 5' BamHI fragment of C11 cDNA, then the 3' fragment, and finally with B10 cDNA insert. The corresponding genomic BamHI bands hybridized by the probes are indicated. In the hybridization with the 5' C11 cDNA probe, there was some cross-hybridization to the 9 kb B10 BamHI band on the Southern, and similarly, with the B10 cDNA probe, there is some cross-reactivity with the 5' C11 BamHI gene fragment at 5.7 kb.

homology between the B10 and C11 cDNAs, restriction maps of the genes do not show a striking overall similarity. One interesting pattern which is shared by the genes is the BamHI/EcoRI/PstI site positions in the upstream region. The promoter elements and perhaps regulatory sequences must be located in this region since hypersensitive sites for both genes occur near the PstI site.

Two genomic clones representing B10 and C11 were isolated by screening with the cDNA inserts. Their identity as genomic C11 and B10 clones was confirmed by a comparison of their restriction enzyme patterns to the map of the genes obtained from genomic DNA. Isolation of the B10 genomic clone has afforded a more extensive restriction map of the B10 gene, for which only a 380 bp cDNA probe was available to probe genomic DNA. A more detailed map of the C11 gene was constructed using a subclone of the genomic C11 clone. By comparing this restriction enzyme map of the gene to the cDNA, a preliminary map of the exon/intron organization was obtained.

The organization of many of the serine protease genes is known, ranging from the ancestral, intron-less bacterial serine protease genes, to the more complex vertebrate genes. The vertebrate serine protease genes have been grouped according to their intron organization, since the genes seem to have evolved by exon shuffling and intron insertion (Rogers, 1985; see figure 6). The first group consists of the haptoglobin gene, which has no introns interrupting the catalytic triad residues. The second group is the trypsin family. In these genes, an intron occurs just downstream of the His codon, another occurs just downstream of the Asp codon and another occurs just upstream of the Ser codon. Therefore, there is one exon to each of the catalytic triad residues and a fourth between the Asp and Ser exons. This is the arrangement of the trypsin, kallikrein and the alpha and gamma nerve growth factor genes. Three variations on this basic pattern exist, which probably arose by intron insertion. One is exemplified by the tissue-type plasminogen activator gene, which has an extra intron just downstream from the Asp codon. Another variation is seen in the chymotrypsin gene, which has two extra introns, one upstream of

the Asp codon and one between the His and activation peptide-encoding sequences. Finally, the elastase gene has the two additional introns of the chymotrypsin gene, plus another intron splitting the exon downstream of the Ser codon. The third family of serine protease genes consists of the complement factor B gene, which contains 7 introns throughout the catalytic region. The fourth group is made up of the factor IX and protein C genes, which have two introns, one separating the sequence encoding the activation peptide and the His codon and one between the His and Asp codons, leaving the Asp and Ser codons on the same exon. Lastly, the thrombin gene comprises the fifth group, in which the catalytic region is split by five exons.

From the deductions made on the placement of the introns in the C11 gene, it appears to belong to the trypsin family. The location of each of the catalytic triad residues on a different exon and the additional exon between the Asp^{88} and Ser^{183}containing exons fulfill the criteria to belong to this group of genes. It does not contain an intron downstream of the Ser^{183} codon, and the Asp^{88} and Ser^{183} codons occur on seperate exons, so it cannot belong to any of the other groups of serine protease genes. However, because not all of the exon/intron boundaries have been determined, the C11 gene could belong to any of the subgroups in the trypsin family except the elastase type. To assign the gene to one of these subgroups, it must be established whether there is an intron between the activation peptide and His^{44}-encoding sequences, whether there are one or two introns between the His^{44} and Asp^{88} codons, and two or three introns between the Asp^{88} and Ser^{183} codons. The proximity of the Asp^{88} codon to the intron boundary downstream of it suggests that the C11 gene falls into the plasminogen activator gene subgroup. Certainly, the overall arrangement of the C11 gene adheres to a general pattern seen in serine protease genes, and in particular the trypsin gene family. It will be interesting to determine the B10 gene organization and whether B10 and C11 are more alike than to other members in the trypsin gene family. The availability of the genomic clones will allow characterization and comparison of the B10 and C11 genes.

The control of expression of B10 and C11 was shown to occur, at least in part, at the level of transcription of the genes. Therefore, the chromatin conformation in the genes was examined using DNase I. The chromatin around transcriptionally active genes is selectively decondensed, which can be assayed by its increased sensitivity to pancreatic DNase I. It is the state of the chromatin, rather than the process of RNA transcription itself, which renders the gene DNase I sensitive, as demonstrated by the fact that even genes which are transcribed only a few times per cell generation are as sensitive as genes which are being actively transcribed. The biochemical basis for the difference in chromatin structure seems to involve a number of things (Weisbrod, 1982). Histone H1, which binds to nucleosomes to pack them, is bound less tightly by active chromatin. The histones are also highly acetylated, which decreases their tendency to pack together *in vitro*. Active genes also bind other non-histone proteins such as HMG14, HMG17 and covalently to ubiquitin. These modifications all seem to contribute to the loosening of the chromatin structure, making it more like "beads-on-a-string" and rendering it DNase I sensitive. The classic examples are globin genes, which are preferentially DNase I-digested in erythrocytes but not in oviduct, and conversely the ovalbumin genes which are selectively digested in chick oviduct but not in erythrocytes (Weintraub and Groudine, 1976; Garel and Axel, 1976).

In addition to an overall DNase I sensitivity, hypersensitive sites have been found in active genes, and these sites correlate with promoter and regulatory regions. Hypersensitive sites seem to consist of localized single-stranded regions of DNA in a nucleosome-free environment. The single-stranded nature of the DNA could be caused by strain introduced by the altered nucleosome conformation or a sequence-specific binding protein (Weisbrod, 1982). DNA footprinting analysis has shown that the sequence bound by a regulatory protein can be 10- to 100-fold more sensitive to DNase I when the protein is bound (Schmitz and Galas, 1979).

The DNase I sensitivity of the B10 and C11 genes was examined in several cell types which differ in their state of expression of the genes. In a thymic cell population, which consists of a mixture of undifferentiated cells committed to the T cell lineage, the genes were not sensitive to DNase I . This implies that the genes are not in a chromatin conformation which would allow their transcription. In the cytotoxic T cell lines, MTL2.8.2 and MTL2.2.1, which are actively transcribing the genes, both the B10 and C11 genes are DNase I sensitive, relative to the inactive globin gene. In addition, hypersensitive sites occur in the upstream region of the genes, which suggest an accessibility to, or binding of, transcription factors. CTL-L16 cells are another CTL line, but which have lost their cytotoxic activity and do not express C11 or B10. However, even in this CTL line, the B10 and C11 genes are DNase I sensitive and the hypersensitive sites are present (although the hypersensitive sites are not present for certain in the C11 gene). Therefore, the genes have the potential to be transcribed, but some inducer molecule is presumably absent. A comparison of DNA footprint analyses (Emerson *et al.*, 1985) using nuclear extracts from the MTL and CTL-L16 lines may provide some interesting information regarding how B10 and C11 gene transcription is induced. EL4 cells, on the other hand, which represent helper T cells, exhibit the overall DNase I sensitivity of the genes relative to a non-expressed gene, but do not possess the hypersensitive sites. The fact that they are sensitive, similar to CTL, but that the thymocytes are not, suggests that T cell differentiation includes an opening up of the chromatin structure around the B10 and C11 genes. *In situ* chromosomal localization of the C11 gene has placed it on mouse chromosome 14 in the vicinity of the T cell antigen receptor genes (Brunet *et al.*, 1986). The decondensation of the chromatin around the B10 and C11 genes in differentiated T cells may be related to the increased accessibility of the T cell antigen receptor genes (Yancopoulos *et al.*, 1986). However, although the hypersensitive sites are not present in the EL4 cells, the 5' region of the C11 gene is more DNase I sensitive than the 3' region of the gene. The difference between the two halves of the gene may be due to a nucleosome-

free area in the 5' region of the gene which unwinds the DNA, making it more susceptible to DNase I attack but not at a highly localized site. The localization of the hypersensitivity to a short region of the gene, which results in formation of a sub-band, may require the binding of a transcription factor, present in T_K, and not in T_H cells. Finally, in a B cell line, S194, the genes are not DNase I sensitive. This again supports the model that the altered chromatin conformation exists only in differentiated T cells.

The relation between the existence of hypersensitive sites, the presence of proteins bound to the site, and state of transcription of the gene varies between different systems. In the case of the beta-globin genes, the hypersensitive sites are tissue- and temporal-specific. A hypersensitive site occurs at the 5' end of the embryonic gene only in embryonic cells, and 5' hypersensitive sites in the adult gene appear in adult cells (Weintraub and Groudine, 1976). In glucocorticoid genes, the hypersensitive site associated with the glucocorticoid response element only appears in the presence of the hormone, and therefore coincides with binding of the regulatory protein to the site (Zaret and Yamamoto, 1984). In heat shock genes, on the other hand, the hypersensitive sites are present even before heat induction (Wu, 1980). However, in this case, proteins are bound at the TATA box, but not in the heat shock regulatory element. An additional protein, heat shock activator protein (HAP), binds the regulatory element upon heat induction and allows transcription to proceed. The gamma-interferon gene has also been assayed for DNase I hypersensitive sites in various cell lines (Hardy *et al.*, 1985). This gene is specifically expressed in helper T cells upon stimulation of the cells with antigen or a mitogen. The hypersensitive site associated with this gene is present in all cells which have the potential to express the gene, even before the cells are activated, similar to the heat shock genes. However, in a variant of the parent cell line which has lost its ability to produce gamma-interferon, the hypersensitive site is no longer present.

The occurence of the hypersensitive sites in the B10 and C11 genes is similar to the pattern for the heat shock and gamma-interferon genes. The sites are present in CTL,

whether or not they are actively transcribing the genes. The site may be due to a nucleosome-free region of the DNA which causes enough conformational strain to unwind it. However, this seems to be the case for the EL4 cells, rendering the 5' region of the gene more susceptible to DNase I degradation than the 3' region, but without causing a specific DNase I hypersensitive site. Formation of the site may require the binding of CTL-specific pre-initiation transcriptional factors, as seen in the heat shock genes. This would also account for the appearance of the sites in the CTL-L16 cells in the absence of transcription. The transcription of the genes would then require an additional factor which binds to a specific regulatory region, and in normal, antigen-dependent T cells, is induced upon antigen-stimulation of the cells, similar to the heat shock activator protein. In fact, C11 induction is blocked by cyclohexamide, implying that a newly synthesized protein is required for gene activation (appendix to chapter 3, section B.3). By analogy, the hypersensitive site in the gamma-interferon gene occurs even in the uninduced cells; therefore a protein which is induced when the cells are stimulated may be required, in addition to other proteins already bound, for transcription to proceed.

The C11 and B10 genes each possess two hypersensitive sites, which by analogy to other genes, will correspond to regulatory regions. In the case of C11, the 2.5 kb sub-band corresponds to a hypersensitive site very near the start of transcription, which probably represents the promoter. The other hypersensitive site is approximately 400 bp upstream of that. For B10, a full-length cDNA is not available, so the site of transcription initiation is not known. However, the 4.5 kb sub-band most probably corresponds to the hypersensitive site at the B10 gene promoter, by comparison to C11. This places the other hypersensitive site 1.2 kb upstream of the promoter. Therefore, the regulatory sites which are furthest 5' to the B10 and C11 genes would have to act over long distances (400 and 1200 bp, respectively), posing the intriguing possibility that they are enhancer sequences. In addition, the overall pattern of the hypersensitive and transcription start sites and the upstream BamHI, EcoRI and PstI sites are remarkably well conserved between the two

genes. Since B10 and C11 are quite homologous and are sequentially expressed in activated CTL, it is quite likely that they share common upstream sequences. The pancreatic serine protease genes have a common enhancer element which regulates their expression (Walker *et al.*, 1983; Brady and MacDonald, 1986). At least two of the T_H lymphokines also share a common element (Hardy *et al.*, 1986). Sequence analysis and comparison of the B10 and C11 genes should reveal whether B10 and C11 share regulatory elements with inducible T cell genes, serine protease genes or CTL-specific genes.

With the isolation of the genomic clones and the identification of potential regulatory sites of the genes by DNase I mapping, the sequences which control expression of B10 and C11 and the proteins which bind at those sites can be identified by DNA footprinting and gel retardation assays (Emerson *et al.*, 1985; Singh *et al.*, 1986). This will help clarify the order of events and molecules necessary for the changes in chromatin configuration related to gene activation and transcription. It will also provide a link between the membrane signalling event which activates CTL and the induction of the B10 and C11 genes. A comparison to other inducible T cell genes may reveal common mechanisms or signalling molecules used to regulate the genes. These approaches will increase our understanding of how the immune system, and in particular the cytotoxic T cell response, is regulated at the molecular level.

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CHAPTER V

CONCLUSIONS

The immune system is comprised of a network of cells, each with a specialized function, which interact to defend the body against invasion. This response to antigen must be finely regulated to optimize the sum of the cells' action, and so that destructive events are directed at the foreign material and not the body's own tissue. Studies at the molecular level are now being used to elucidate how the regulation and functions of the immune system are achieved. The molecular genetic approach described here has focussed on the mechanism by which a cytotoxic T cell (CTL, T_K) lyses its target cell and how this response may be regulated.

Initially, the genes encoding molecules which may be important to CTL function were isolated by a differential approach. This method is based on the assumption that proteins which are required in a cell's specialized function are represented by mRNAs present in that cell type, but at a low level or not at all in other types of cells. Thus, cytotoxic T lymphocytes should express a set of mRNAs that encode its function-related proteins and that are not present in helper T cells (T_H) or thymocytes. To isolate such mRNA sequences, a cDNA library was constructed using mRNA from a CTL line as template. This library was sequentially screened with probe generated from T_K, T_H and thymus cells. Several cDNA clones were identified which were positive with the T_K and not the other probes. Two of these, B10 and C11, were characterized in detail.

The specificity of expression of these two clones was tested in a number of different cell types. Of 12 T cell lines or cultures tested which had cytotoxic activity, all 12 expressed B10 and C11. Surprisingly, 4 peritoneal exudate lymphocyte (PEL) lines were negative for the clones, although they are cytotoxic cells. These cells, however, apparently do not possess the characteristic CTL granules, nor the complement-like perforin molecules. Therefore they are believed to lyse their targets by a mechanism different than

that used by CTL. Natural killer (NK) cells, which do possess granules and are thought to kill their target cells using a similar mechanism to CTL, also did not express detectable B10 or C11 transcripts and so possibly utilize some other B10- or C11-like molecules instead. Indeed, another DNA clone, gN1, that was isolated by its high degree of homology to C11, is expressed specifically in NK cells. Other types of lymphoid cells tested included 5 non-cytolytic helper T cell lines, resting and activated splenic B cells and macrophages, all of which were negative for B10 and C11 transcripts. The non-lymphoid cells tested, brain, liver and fibroblasts, did not express B10 or C11. Thus, these two clones, which were identified by differential hybridization screening, represent mRNAs which are only expressed in T cells with cytolytic activity. The only exception was the helper T cell clone, D10, which expresses C11 but does not express B10.

The expression of B10 and C11 was found to be not only CTL-specific, but also to correlate with the activation of cytotoxicity. In time course experiments, the level of B10 and C11 mRNA increased when antigen-dependent cytotoxic T cell lines or spleen cell cultures were activated by antigen or ConA. The peak of induction of C11 mRNA occurred first, followed by B10 mRNA, and finally, the peak of cytotoxic activity. This order of events occurred regardless of whether the cell's response peaked in a few hours or in a few days. Thus, these two mRNAs seem to be sequentially regulated in response to CTL activation. The CTL-specific expression and the correlation with T cell cytotoxic activity strongly suggest that the B10- and C11-encoded proteins play a key role in CTL function.

The induction of C11 expression upon cell activation was not inhibited by phorbol myristate acetate (PMA) in a memory CTL response or cyclosporin A (CsA) in an antigen-dependent response, although these two compounds inhibited cytotoxic activity of the cultures. Therefore, these drugs must exert their effects on some other molecules which are critical to the lytic event, or at a point after the B10 and C11 induction signals have occurred. C11 induction can, however, be blocked by cyclohexamide. This implies that

the induction signal requires *de novo* protein synthesis. Lymphotoxin, another molecule implicated in target cell destruction, is induced with the same kinetics as C11, but this induction is not blocked by cyclohexamide. Therefore the two genes must be activated by a different signal. By using B10 and C11 as probes, together with other cloned genes which are induced in T cells, these sorts of analyses should help to clarify the order of events between membrane signalling and target cell lysis.

Having established the CTL-specificity of B10 and C11, the nature of the encoded proteins and their possible role in cytolysis was determined. Sequence analysis of B10 and C11 revealed that they were related to each other (80% homologous at the nucleotide level) and that both appear to encode serine proteases. In addition to the overall homology with this class of enzymes, the C11-encoded protein contains the residues which form the catalytic triad in the active site of all known serine proteases (His^{57}, Asp^{102} and Ser^{195} in chymotrypsin). This protein was designated "cytotoxic cell protein" (CCPI). The B10 cDNA insert was not full-length, but it does include the Ser^{195} region and has a high degree of homology to C11. This suggests that it, too, encodes a serine protease, referred to as CCPII. By homology to other serine proteases, CCPI includes a 19 amino acid signal sequence and an unusually short activation peptide of 2 amino acid residues, Gly-Glu. The serine protease which CCPI shares the greatest homology with, rat mast cell protease II (RMCPII), possesses some very unusual characteristics which affect substrate binding (Woodbury, 1980). CCPI shares many of these characteristics, suggesting that it has a novel substrate specificity.

Synthetic peptides based on the C11 sequence were synthesized and used to generate antibodies against CCPI. The antibodies were used for cell localization of CCPI and Western blots, which demonstrated that CCPI exists in the granules of CTL. This supported the idea that the C11-product is involved in the lytic event, since granules are believed to contain the effector molecules for CTL lysis. At least two species of serine proteases were suspected to exist in the CTL-specific granules, identified by

diisopropylflourophosphate (DFP)-binding. One of these runs at 60 kD on a non-reducing and 35 kD on a reducing gel, while the other runs at 29 kD both under reducing and non-reducing conditions. The Western blot analysis using the CCPI antibodies demonstrated that the C11-encoded product corresponds to the 29 kD protein. As we had predicted from the C11 sequence, this protein is reported to have an unusual substrate-specificity.

Taken together, the above data provides strong evidence that B10 and C11 represent molecules which are directly involved in CTL function. Both are specifically expressed in T cells with cytotoxic activity and their expression correlates with the level of lytic activity of the cells. At least one of the encoded proteins exists in the granules, which possess the cells' lytic activity. The fact that CCPI, and possibly CCPII, are serine proteases suggests possible roles these molecules may play in the lytic event. They could be involved in a cascade-type reaction leading to polymerization of perforin, similar to complement activation. Alternatively, they may be involved directly in target cell destruction by attacking cell membrane proteins or passing through the perforin channels to cleave a substrate(s) inside the target cell.

The regulation of expression of these two molecules was next examined. The induction of C11 and B10 expression was found to occur, at least in part, at the level of transcription (J. Shaw, unpublished). Therefore, the genes corresponding to B10 and C11 were isolated and characterized, as a first step in studying how they are regulated.

By probing Southern blots of genomic DNA and analyzing genomic clones (gB10 and gC11), a restriction enzyme map of the two genes was constructed. Comparison of the map of gC11 to the C11 cDNA map allowed a preliminary assignment of the intron positions in the gene. Many serine protease genes have been mapped, and have been divided into groups, according to their gene organization, which are thought to reflect their evolutionary background. Comparison of the C11 gene organization to other serine protease genes showed that it shares the exon/intron arrangement of the trypsin gene family. It will be interesting to compare the gene organization of B10, gN1, D12,

RMCP(II) and cathepsin G, which seem to comprise a multigene family together with C11.

Possible regulatory regions of the B10 and C11 genes were identified by analyzing the chromatin configuration around them. The chromatin in the region of genes which have the potential to be transcribed is relatively decondensed. This altered conformation renders different genes in different cell types accessible to transcription factors, and thus is a first step in cell-specific gene regulation. The loosening of the chromatin can be detected using low levels of DNase I, which preferentially degrades the DNA in the accessible chromatin. Within these DNase I sensitive regions, hypersensitive sites occur due to strain induced by nucleosome-free areas in the DNA. These hypersensitive sites have been found to correspond to regulatory regions, and can become 10- to 100-fold more sensitive when the corresponding regulatory protein binds the site. The DNase I sensitivity of the B10 and C11 genes was examined in three CTL lines, two of which are actively transcribing the gene and a variant CTL line which has lost both cytotoxic activity and the ability to transcribe B10 and C11. For comparison, the sensitivity of the genes in thymus cells, which consist of a heterogeneous population of undifferentiated T cells, a line representing helper T cells, and a B cell line were also examined.

In the undifferentiated T cells of the thymus, the genes were not DNase I sensitive, and presumably not available for transcription. However, differentiation to functional T cells, whether of the helper or killer subset, seemed to be sufficient to confer an overall DNase I sensitivity to the genes. This may be related to the decondensation around the T cell antigen receptor genes, since the C11 gene has been localized to the same region of the mouse chromosome 14 gene. In addition, the 5' half of the C11 gene is more sensitive than the 3' half in differentiated T cells, suggesting that a nucleosome-free region exists there. In this case, however, the killer and helper T cells differ. In killer T cells, sub-bands appear as the main bands are digested, which correspond to DNase I hypersensitive sites in the upstream region of the genes. In the helper T cells, however, there is a random nicking of the gene and no hypersensitive site. Formation of the hypersensitive sites may

require the binding of CTL-specific transcription factors. These factors, however, are not sufficient to induce transcription, since the CTL line which no longer expresses C11 and B10 possesses the hypersensitive sites. Therefore, another factor may be induced upon CTL-activation which must bind in order for transcription to proceed. In accordance with this, cyclohexamide blocks the induction of C11 and B10 mRNA, presumably by blocking synthesis of an induced transcription factor. Finally, in the B cell line tested, the B10 and C11 genes exhibited no DNase I sensitivity, as expected if chromatin decondensation correlates with T cell differentiation. A model for the activation of the B10 and C11 genes, then, is that during T cell differentiation the chromatin around the B10, C11 and other T cell-specific genes becomes relatively decondensed, and the 5' regions of the B10 and C11 genes contain a nucleosome-free area. A CTL-specific, pre-initiation transcription factor binds the DNA, which leads to the formation of the hypersensitive sites. Upon activation of CTL, another transcription factor(s) is induced and binds to the regulatory regions of the B10 and C11 genes, thereby allowing transcription to proceed. A similar order of events may occur for helper T cell inducible genes, as indicated by the gene for gamma-interferon.

A comparison of the regulatory DNA sequences of the B10 and C11 genes which occur in the vicinity of the DNase I hypersensitive sites, as well as the regulatory sequences of other genes activated in T cells may reveal some common elements used by these genes. Identification of the regulatory factors of the genes will help elucidate the events which occur between antigen-activation at the cell surface, and gene activation in the T cell nucleus. This may, in turn, provide information on how the genes induced in T cells are coordinately and sequentially regulated.

In addition to B10 and C11, two other interesting DNA sequences were isolated and characterized. Both were identified by their high degree of homology with C11. Preliminary sequence information for one of these, D12, suggests that it too may encode a serine protease. Its expression appears to be limited to the recently characterized cytotoxic-helper T cells and to antigen-independent T cell lines. Another clone, gN1, is NK-specific

and may correspond to a CCPI-equivalent in those cells, since C11 and B10 are not expressed in them.

In summary, two gene sequences which are specifically expressed in CTL were isolated from a CTL cDNA library by differential screening, and the level of their expression found to correlate with T cell cytotoxic activity. Both appear to encode serine proteases and one of these has been localized to the characteristic CTL granules which possess the lytic activity of the cells. Taken together, this data strongly suggests that these molecules play an important role in target cell lysis by CTL. Regulation of their expression occurs at the level of transcription. DNase I assays suggest that the B10 and C11 genes are controlled, first by a loosening of the chromatin around the genes in differentiated T cells, then by the binding of a CTL-specific factor which leads to the formation of hypersensitive sites, and finally by the binding of an inducible factor which allows transcription to proceed. This would account for the CTL-specific and activation-dependent expression of the B10 and C11 genes.

The CTL-specific genes which have been isolated represent useful markers of cell function. Unlike most T cell markers, which are cell-surface antigens of unknown function, B10 and C11 expression appears to be associated with T cell cytotoxic activity. These markers will be useful, not only in assigning cells to a T cell subset, but also in determining the role of CTL in immune defense and in autoimmune dysfunction. The identification of molecules which seem to be directly involved in CTL lysis has increased our understanding of how the cells may induce target cell destruction. Characterizaton of the molecules involved in the lytic event will allow the development of specific potentiators or inhibitors of CTL function. The novel substrate specificity of CCPI may be useful, for example, in developing synthetic substrates which selectively block the CTL response, without inhibiting other important physiological functions. The ability to monitor the role of CTL in disease, together with a knowledge of how the cells function and are regulated at the molecular level, provides a basis for the development of rational immunotherapy.